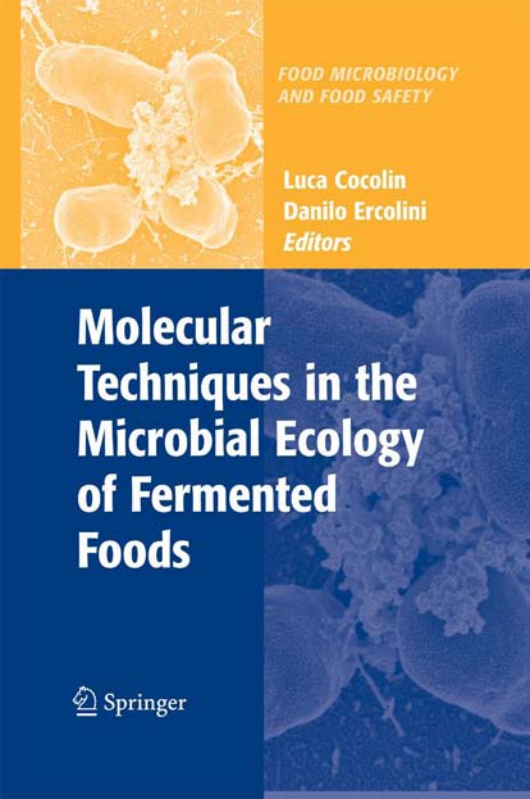


The book cover features a background image of a microscopic view of bacteria. The top half has an orange background with a cluster of rod-shaped bacteria. The bottom half has a dark blue background with a cluster of spherical bacteria. The title and editor information are overlaid on these images.

*FOOD MICROBIOLOGY
AND FOOD SAFETY*

Luca Cocolin
Danilo Ercolini
Editors

Molecular Techniques in the Microbial Ecology of Fermented Foods

 Springer

VISIT...

LANZAROTE
Caliente.COM

Molecular Techniques in the Microbial Ecology of Fermented Foods

FOOD MICROBIOLOGY AND FOOD SAFETY SERIES

Food Microbiology and Food Safety publishes valuable, practical, and timely resources for professionals and researchers working on microbiological topics associated with foods as well as food safety issues and problems.

Series Editor

Michael P. Doyle, *Regents Professor and Director of the Center for Food Safety, University of Georgia, Griffin, GA, USA*

Editorial Board

Francis F. Busta, *Director – National Center for Food Protection and Defense, University of Minnesota, Minneapolis, MN, USA*

Bruce R. Cords, *Vice President, Environment, Food Safety & Public Health, Ecolab Inc., St. Paul, MN, USA*

Catherine W. Donnelly, *Professor of Nutrition and Food Science, University of Vermont, Burlington, VT, USA*

Paul A. Hall, *Senior Director Microbiology & Food Safety, Kraft Foods North America, Glenview, IL, USA*

Ailsa D. Hocking, *Chief Research Scientist, CSIRO – Food Science Australia, North Ryde, Australia*

Thomas J. Montville, *Professor of Food Microbiology, Rutgers University, New Brunswick, NJ, USA*

R. Bruce Tompkin, *Formerly Vice President-Product Safety, ConAgra Refrigerated Prepared Foods, Downers Grove, IL, USA*

Titles

PCR Methods in Foods, John Maurer (Ed.) (2006)

Foodborne Parasites, Ynes R. Ortega (Ed.) (2006)

Viruses in Foods, Sagar Goyal (Ed.) (2006)

Molecular Techniques in the Microbial Ecology of Fermented Foods, Luca Cocolin and Danilo Ercolini (Eds.) (2008)

Luca Cocolin • Danilo Ercolini

Editors

Molecular Techniques in the Microbial Ecology of Fermented Foods

 Springer

Luca Cocolin
Dipartimento di Valorizzazione
e Protezione delle Risorse Agroforestali
University of Torino
Italy

Danilo Ercolini
Department of Food Science
School of Biotechnological Sciences
University of Naples Federico II
Italy

Advisory Board for this current work:

Professor Salvatore Coppola
University of Naples Federico II
Italy

Dr. Kalliopi Rantsiou
University of Torino
Italy

ISBN: 978-0-387-74519-0

e-ISBN: 978-0-387-74520-6

Library of Congress Control Number: 2007936620

© 2008 Springer Science+Business Media, LLC

All rights reserved. This work may not be translated or copied in whole or in part without the written permission of the publisher (Springer Science+Business Media, LLC, 233 Spring Street, New York, NY 10013, USA), except for brief excerpts in connection with reviews or scholarly analysis. Use in connection with any form of information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed is forbidden.

The use in this publication of trade names, trademarks, service marks, and similar terms, even if they are not identified as such, is not to be taken as an expression of opinion as to whether or not they are subject to proprietary rights.

Printed on acid-free paper.

9 8 7 6 5 4 3 2 1

springer.com

Preface

The approach to study microorganisms in food has changed. In the last few years the field of food fermentations has experienced a very fast development, thanks to the application of methods allowing precise picturing of their microbial ecology. As a consequence, new information is available on the structure and dynamics of the microbial populations taking turns during fermented food production.

This is the age when functional genomics, transcriptomics, proteomics and metabolomics are going to shed light on the overall role of bacteria in food fermentation, considering also their interactions. Nevertheless, the last 10 years can be considered the “detectomics” era, since much research effort has been dedicated to the development and optimization of biomolecular methods for the detection, reliable identification and monitoring of microorganisms involved in food fermentations. The identification of species and strains during the different phases of fermented foods production allows the understanding of the time when they act or play a role in the food matrix, and the molecular methods can, thus, be used for this purpose in a sort of functional diagnostics.

It is well recognized by researchers world-wide that traditional microbiological methods often fail to characterize minor populations or microorganisms for which a selective enrichment is necessary. Moreover, stressed and injured cells need specific culturing conditions to recover and become cultivable on agar media. Lastly, conventional microbiological techniques are not able to detect viable, but not culturable, cells. The use of molecular techniques allows the precise study of the microbial populations involved in the food fermentation, avoiding the biases related with the traditional methods.

This book takes into consideration both well-known fermented foods and non-European foods and describes the latest findings in the microbial ecology as determined by the application of molecular methods. Culture-dependent techniques, defined as identification, molecular characterization and typing of microbes isolated from the food, and culture-independent methods, as description of the microbial populations present (at DNA level) and/or active (at RNA level) without the need of traditional isolation, are taken into consideration.

All the fermentations are dealt with, including dairy, meat, cereal, wine, beer and vegetables, as well as other fermentations such as those for the production of Asian

and South American products. Moreover, critical chapters on the use of 'omics' in food fermentation and on molecular techniques to study probiotic bacteria and gut ecology are included. Finally, two chapters are respectively dedicated to the methods and their technical aspects, and to the use of bioinformatics for the analysis of sequencing data.

The subject is approached in a way that provides the reader with analytical details and suggestions useful in research, as well as criticism in the evaluation of the benefits that can arise by using novel approaches in food fermentation microbiology. The philosophy of the book is to report the most recent advances in the field, and researchers will find details on primers and protocols most suitable for studying their specific food ecosystem. Apart from the research scopes, the book will allow students of different levels to approach the subject and will provide knowledge on the microbiology of fermented foods to allow an early awareness of how certain food processes are studied today.

The above is the overall plot, beyond which we gave the contributors wide autonomy to set about their own subjects with the appropriate contents and criticism. A team of international scientists, experts in the different food fermentations, have contributed to this volume.

A number of books are available on the microbiology of fermented foods, but this is the first to approach the subject from a novel point of view, reporting the new insights drawn in the microbial ecology of fermented foods by using bio-molecular techniques.

Naples, June 1, 2007

Luca Cocolin and Danilo Ercolini

Contents

Preface	v
List of Contributors	ix
Chapter 1 Molecular Techniques in Food Fermentation: Principles and Applications	1
Giorgio Giraffa and Domenico Carminati	
Chapter 2 Dairy Products	31
Salvatore Coppola, Giuseppe Blaiotta, and Danilo Ercolini	
Chapter 3 Fermented Meat Products	91
Kalliopi Rantsiou and Luca Cocolin	
Chapter 4 Sourdough Fermentations	119
Rudi F. Vogel and Matthias A. Ehrmann	
Chapter 5 Vegetable Fermentations	145
Hikmate Abriouel, Nabil Ben Omar, Rubén Pérez Pulido, Rosario Lucas López, Elena Ortega, Magdalena Martínez Cañamero, and Antonio Gálvez	
Chapter 6 Wine Fermentation	162
David A. Mills, Trevor Phister, Ezekial Neeley, and Eric Johannsen	
Chapter 7 Beer Production	193
Giuseppe Comi and Marisa Manzano	
Chapter 8 Other Fermentations	208
Christèle Humblot and Jean-Pierre Guyot	

Chapter 9	Probiotics: Lessons Learned from Nucleic Acid-Based Analysis of Bowel Communities	225
	Rodrigo Bibiloni, Christophe Lay, and Gerald W. Tannock	
Chapter 10	Bioinformatics for DNA Sequence-based Microbiota Analyses.	245
	Knut Rudi	
Chapter 11	Role of Bacterial ‘Omics’ in Food Fermentation.	255
	Monique Zagorec, Stéphanie Chaillou, Marie Christine Champomier-Vergès, and Anne-Marie Crutz – Le Coq	
Index		275

List of Contributors

Abriouel Hikmate

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas s/n. 23071-JAEN,
Spain

Ben Omar Nabil

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas s/n. 23071-JAEN,
Spain

Bibiloni Rodrigo

Department of Microbiology and Immunology, University of Otago, PO Box 56,
Dunedin, New Zealand

Blaiotta Giuseppe

School of Agriculture, Department of Food Science, University of Naples
Federico II, Via Università 100, 80055 Portici, Naples, Italy

Carminati Domenico

C.R.A. – Istituto Sperimentale Lattiero Caseario, Via Lombardo 11, 26900 Lodi,
Italy

Chaillou Stéphanie

Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert,
F-78350 Jouy-en-Josas, France

Champomier-Vergès Marie Christine

Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert,
F-78350 Jouy-en-Josas, France

Cocolin Luca

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali,
University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco – Torino, Italy

Comi Giuseppe

Dipartimento di Scienze degli Alimenti, University of Udine, Via Marangoni 97,
33100 Udine, Italy

Coppola Salvatore

School of Agriculture, Department of Food Science, University of Naples
Federico II, Via Università 100, 80055 Portici, Naples, Italy

Crutz – Le Coq Anne-Marie

Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert,
F-78350 Jouy-en-Josas, France

Ehrmann Matthias A.,

Lehrstuhl für Technische Mikrobiologie, Technische Universität München,
Weihenstephaner Steig 16, D-85350
Freising-Weihenstephan, Germany

Ercolini Danilo

School of Biotechnological Sciences, Department of Food Science, University
of Naples Federico II, Via Università 100, 80055 Portici, Naples, Italy

Gálvez Antonio

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas
s/n. 23071-JAEN, Spain

Giraffa Giorgio

C.R.A. – Istituto Sperimentale Lattiero Caseario, Via Lombardo 11, 26900 Lodi,
Italy

Guyot Jean-Pierre

Institut de Recherche pour le Développement (IRD), BP 64501, 34394
Montpellier cedex 5, France

Humblot Christèle

Institut de Recherche pour le Développement (IRD), BP 64501, 34394
Montpellier cedex 5, France

Johannsen Eric

Department of Viticulture & Enology, University of California, One Shields
Avenue, Davis, CA 95616-8749 U.S.A and LaCrema Winery, 3690 Laughlin
Road, Windsor, CA 95492 U.S.A

Lay Christophe

Department of Microbiology and Immunology, University of Otago, PO Box 56,
Dunedin, New Zealand

Lucas López Rosario

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología, Fac.
Ciencias Experimentales, Campus Las Lagunillas
s/n. 23071-JAEN, Spain

Manzano Marisa

Dipartimento di Scienze degli Alimenti, University of Udine, Via Marangoni 97,
33100 Udine, Italy

Martínez Cañamero Magdalena

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas
s/n. 23071-JAEN, Spain

Mills David A.

Department of Viticulture & Enology, University of California, One Shields
Avenue, Davis, CA 95616-8749 U.S.A

Neeley Ezekial

Department of Viticulture & Enology, University of California, One Shields
Avenue, Davis, CA 95616-8749 U.S.A and Bonny Doon Vineyards, 10 Pine Flat
Road, Santa Cruz, CA 95060 U.S.A

Ortega Elena

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas
s/n. 23071-JAEN, Spain

Pérez Pulido Rubén

University of Jaen, Dpto. Ciencias de la Salud, Area de Microbiología,
Fac. Ciencias Experimentales, Campus Las Lagunillas
s/n. 23071-JAEN, Spain

Phister Trevor

Department of Food Science, North Carolina State University, 100 Schaub Hall,
Campus Box 7624, Raleigh, NC 27695-7624 U.S.A

Rantsiou Kalliopi

Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali,
University of Torino, Via Leonardo da Vinci 44, 10095 Grugliasco – Torino, Italy

Rudi Knut

Matforsk AS, Norwegian Food Research Institute, Ås, Norway;
Hedmark University College, Hamar, Norway

Tannock Gerald

Department of Microbiology and Immunology, University of Otago, PO Box 56,
Dunedin, New Zealand

Vogel Rudi F.

Lehrstuhl für Technische Mikrobiologie, Technische Universität München,
Weihenstephaner Steig 16, D-85350 Freising-Weihenstephan, Germany

Zagorec Monique

Unité Flore Lactique et Environnement Carné, INRA, Domaine de Vilvert,
F-78350 Jouy-en-Josas, France

Chapter 1

Molecular Techniques in Food Fermentation: Principles and Applications

Giorgio Giraffa and Domenico Carminati

Abstract The dynamics of growth, survival, and biochemical activity of microorganisms in fermented foods are the result of stress reactions in response to the changing of the physical and chemical conditions into the food micro-environment, the ability to colonize the food matrix and to grow into a spatial heterogeneity, and the *in situ* cell-to-cell ecological interactions which often happen in a solid phase. To this regard, estimates of true microbial diversity in fermented food products are often difficult chiefly on account of the inability to cultivate most of the viable bacteria or to evaluate stressed cells. Traditional methods of microbial enumeration, identification, and characterization are insufficient for monitoring specific strains in complex, mixed-strain microbial communities. In the last decade, due to the use of molecular methods, our knowledge about the microbial diversity of microbial ecosystems has dramatically increased. In particular, new and highly performing culture-independent and culture-dependent molecular techniques are now available to study food-associated microbial communities. While the former are helping to afford peculiar problems related to composition and population dynamics of heterogeneous microbial communities in complex food matrices, the latter are expanding our knowledge about taxonomic diversity of the food-related microflora. Molecular approaches to study the evolution of microbial flora could be useful to better comprehend the microbiological processes involved in food processing and ripening, improve microbiological safety by monitoring *in situ* pathogenic bacteria, and evaluate the effective composition of the microbial populations. In this chapter, a general overview of molecular methods to study microbial populations in food fermentation will be given. Recent advances and technical description of these methods will be outlined.

Keywords microbial ecology; food ecosystems; lactic acid bacteria; molecular techniques; food fermentation

1 Introduction

Within the fermentation industry, microorganisms are used for the production of specific metabolites such as acids, alcohols, enzymes, antibiotics and carbohydrates. Major fermentation microbes include lactic acid bacteria (LAB), molds and yeasts. In particular, LAB are the major microflora involved in fermented dairy products, vegetable, and sourdough fermentation, and (mainly lactobacilli and pediococci) are part of the starter cultures used in meat fermentation to produce desirable acids and flavor compounds. Industrial control of fermentation processes requires up-to-date knowledge of the physiology, metabolism and genetic properties of such microorganisms.

Searching for the presence, numbers, and types of microorganisms in foods is of paramount importance for the food industry. There are three major applications: (i) identifying the bacterial flora of starter cultures and foods; (ii) determining the total numbers of bacteria in food samples, and (iii) detecting particular strains and/or biotypes in food products. However, quality and safety assurance are equally important elements in food production, with food increasingly having to meet the market's stringent requirements. Therefore, it is also important to detect hazardous or unwanted microorganisms, such as bacteria, viruses, yeasts and molds if they are present in the product.

Whatever the primary objective of these microbial analyses (e.g. control of food quality, food preservation, efficiency of starter cultures, monitoring of particular species/strains), the taxonomic level of the microbial discrimination needed should be initially decided. In diagnostic microbiology, this taxonomy depends upon the sensitivity of the technique (either phenotypic or genotypic) used and may range from genus (or species) to subspecies or strain level (sub-typing). However, evaluating microbial diversity in fermented food is problematic because it is often difficult to cultivate most of the viable bacteria or to detect stressed cells. This led to the introduction of new and highly performing molecular methods to study food-associated microbial communities. The focus of this chapter is to give a general overview of molecular methods (both culture-independent and culture-dependent) to study microbial populations in food fermentation. Recent advances and technical description of these methods will also be outlined.

2 The Qualitative and Quantitative Estimation of Microbial Populations in Fermented Food: Problems and Needs

A food ecosystem is not static. The dynamics of growth, survival and biochemical activity of microorganisms in foods are the result of stress reactions in response to changing physical and chemical conditions that occur in the food micro-environment (e.g. pH, salt, temperature), the ability of microorganisms to colonize the food matrix and to grow into spatial heterogeneity (e.g. micro-colonies and biofilms),

and the *in situ* cell-to-cell ecological interactions which often happen in a solid phase. Reliable quantitative microbiological data should, therefore, take into consideration the dynamics of microorganisms in food ecosystems. This information is of key importance in food ecology, especially in understanding the behavior of pathogens and LAB in foods (Fleet 1999).

2.1 *Survival Mechanisms and Stress Reactions*

It is widely accepted that plate culturing techniques reveal little of the true microbial population in natural ecosystems. This phenomenon can be explained by two main factors:

- the inability to detect novel microorganisms, which might not be cultivable using known media;
- the inability to recover known microorganisms which are either stressed or enter a viable but non-cultivable (VBNC) state (Fleet 1999).

The VBNC state is induced when adverse conditions such as nutrient depletion, low temperature and stresses such as pH and heat treatments can cause healthy, cultivable cells to enter a phase in which they are still capable of metabolic activity, but do not produce colonies on media (both non-selective and selective) that normally support their growth. The VBNC state has been shown in both Gram positive and Gram negative microbial species in the natural environment, and it has also been experimentally induced in most food-borne pathogens (Roszak and Colwell 1987; Fleet 1999) and *Enterococcus faecalis* (del Mar Lleo, et al. 2000).

2.2 *In situ Reactions and Microbial Communication*

The discovery that bacteria are able to communicate with each other changed our general perception of many single, simple organisms inhabiting our world. Instead of language, bacteria use signaling molecules, which are released into the environment. A wide range of communication mechanisms have been described so far within bacteria, such as production of bacteriocins, pheromones, and signaling molecules (e.g. acyl-L-homoserine lactones). As well as releasing the signaling molecules, bacteria are also able to measure the number (concentration) of the molecules within a population. Today we use the term 'Quorum Sensing' (QS) to describe the phenomenon whereby the accumulation of signaling molecules enable a single cell to sense the number of bacteria (cell density) (Konaklieva and Plotkin 2006).

Quorum sensing enables bacteria to coordinate their behavior. Environmental conditions often change rapidly, and bacteria need to respond quickly to survive. These responses include adaptation to available nutrients, defense against other

microorganisms – which may compete for the same nutrients – and avoiding toxic compounds that are potentially dangerous for the bacteria. Today, several QS systems are intensively studied in various organisms such as marine bacteria and some pathogenic bacteria. Quorum sensing is very important for pathogenic bacteria during infection of a host (e.g. humans, other animals or plants) to co-ordinate their virulence. Although little is still known on the role of QS in food ecosystems, it has recently been shown that this mechanism regulates the *in situ* phenotypic expression and population behavior of food spoilage bacteria (Gram, et al. 2002).

In response to the above needs, genetic methods based upon molecular biology have been developed recently to study microbial populations without cultivation and for the identification and sub-typing of cultivable bacteria. Today, a number of molecular techniques can provide outstanding tools for the detection, identification, and characterization of bacteria involved in fermented food processes (Giraffa 2004; Rantsiou and Coccolin 2006). In deciding to offer a routine service based upon one or more of these techniques – type-ability, reproducibility, discriminating power, ease of use, reliability, automation and cost – should all be taken into consideration.

3 Culture-independent Techniques

To determine the diversity of microorganisms in natural ecosystems and to monitor the evolution of microbial populations over space or time, culture-independent methods have been developed. Compared to traditional culturing, these methods aim to obtain a picture of a microbial population without the need to isolate and culture its single components. This is possible because these techniques are based upon a “community DNA/RNA isolation approach.” Although there are limitations to these methods, they can, nevertheless, be very useful once these limitations are taken into consideration (for a review, see Forney, et al. 2004). Such limitations include technical problems, such as obtaining representative genomic DNA from food samples, to conceptual questions, such as using universally accepted and meaningful definitions of microbial species. Culture-independent techniques and their application to fermented food have been reviewed (Giraffa and Neviani 2001; Ercolini 2004); the most commonly applied methods are reported in Table 1.1.

3.1 PCR-based Methods

PCR has revolutionized microbial ecology, resulting in the development of several techniques of microbial community fingerprinting. Although most of these methods are generally based on the amplification of only the variable regions or the totality of the 16S rRNA genes, amplified fragments can also derive from total RNA extracted from food and amplified by reverse transcriptase-PCR (RT-PCR).

Table 1.1 Summary of the Most Widely Used Culture-independent Techniques and Their Applications to Microbial Ecology

	Taxonomic Resolution	Applications to Microbial Ecology
PCR-based Methods		
- PCR-DGGE/PCR-TGGE	Community members (genus/species level)	Community fingerprinting; population dynamics
- SSCP	Community members (genus/species level)	Mutation analysis; community fingerprinting; population dynamics
- T-RFLP	Community and population members (genus, species, strain level)	Community fingerprinting; dynamics between (species-dynamics) and within (strain-dynamics) populations
- LH-PCR	Community members (genus/species level)	Community fingerprinting; population dynamics
- PCR-ARDRA	Community members (species level)	Automated assessment of microbial diversity within communities of isolated microorganisms
- RISA/ITS-PCR	Particular community members (species groups level)	Community fingerprinting; population dynamics
- AP-PCR	Population members (strain level)	Automated estimation of diversity (typing) within populations
- AFLP	Community and population members (genus, species, and strain level)	Automated estimation of diversity within communities (species composition) and populations (typing)
<i>In situ</i> Methods		
- FISH	Community members (species level)	Detection of viable (both cultivable and uncultivable) cells within communities; temporal and spatial distribution of microbes within ecosystems
- Multiplex FISH	Community members (species level)	Similar to FISH; simultaneous investigation of complex communities (e.g. biofilms)
- Fluorescence <i>in situ</i> PCR	Community members (species level)	Detection of viable, slow-growing cells within communities; sensitive identification of target sequences with low copy number
Other methods		
- Flow cytometry	Population members (strain level)	Selective enumeration of mixed microbial populations and sub-populations; physiological cell state analysis.

(continued)

Table 1.1 Summary of the Most Widely Used Culture-independent Techniques and Their Applications to Microbial Ecology (continued)

	Taxonomic Resolution	Applications to Microbial Ecology
- Competitive PCR	Community members (species level)	Detection of cells into the VNC state
- Quantitative hybridization	Community members (species level)	(Semi)quantitative population dynamics of physiologically-active microbial groups

Acronyms legend: PCR-DGGE/TGGE, PCR-Denaturing Gradient Gel Electrophoresis/Thermal Gradient Gel Electrophoresis; SSCP, Single Strand Conformation Polymorphism; T-RFLP, Terminal-Restriction Fragment Length Polymorphism; PCR-ARDRA, PCR-Amplification Ribosomal DNA Restriction Analysis; RISA/ITS-PCR, rRNA gene Internal Spacer Analysis/Intergenic Transcribed Spacer-PCR; AP-PCR, Arbitrarily Primed-PCR; AFLP, adaptor fragment length polymorphism; FISH, Fluorescence *in situ* hybridization.

Since active bacteria have a higher number of ribosomes than dead cells, the use of RNA instead of DNA highlights the metabolically active populations present in the ecosystem. PCR methods are rapid, easy to use, inexpensive, and moderately reproducible. Nevertheless, biases inherent in any PCR amplification approach – such as preferential annealing to particular primer pairs, or an incidence of chimeric PCR products with increasing numbers of PCR cycles – should be carefully evaluated and resolved to improve the reliability of quantitative predictions (Suzuki and Giovannoni 1996; Wang and Wang 1997; Wintzingerode, et al. 1997; Sánchez, et al. 2006).

PCR-denaturing gradient gel electrophoresis (PCR-DGGE) and PCR-temperature gradient gel electrophoresis (PCR-TGGE) were introduced 10 years ago in environmental microbiology and are now routinely used in many laboratories worldwide as molecular methods to study population composition and dynamics in food-associated microbial communities. These two techniques essentially consist of the amplification of the genes encoding the 16S rRNA from the matrix containing different bacterial populations, followed by the separation of the DNA fragments. Separation is based on the decreased electrophoretic mobility of PCR amplified, partially melted, double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (PCR-DGGE) or a linear temperature gradient (PCR-TGGE). Molecules with different sequences may have different melting behavior and will stop migrating at different positions along the gel. The PCR-DGGE (or PCR-TGGE) generated patterns could provide a preliminary ecological view of predominant species increasing or decreasing in complex microbial communities by observing appearance or disappearance of specific amplicons in the denaturing gel (Muyzer, et al. 1993; Felske, et al. 1998).

PCR-DGGE has been widely applied to several fields of food microbiology: the identification of microorganisms isolated from food, the assessment of the impact of probiotic bacteria on the native human gastrointestinal microflora, the evaluation of microbial diversity during food fermentation (e.g. naturally fermented Sausages,

dairy and cereal products, wine, rice vinegar), and the assessment of the microbiological and commercial food quality (Walter, et al. 2000; Lopez, et al. 2003; Ercolini 2004; Giraffa 2004; Fontana, et al. 2005; Haruta, et al. 2006; Rantsiou and Coccolin 2006) represent some examples. Although the 16S rRNA gene offers the benefits of robust database and well-characterized phylogenetic primers, PCR-DGGE (or PCR-TGGE) analyses could not be limited to ribosomal gene markers, which often present intraspecies heterogeneity. To overcome this limitation, the use of different phylogenetic markers [e.g. genes coding for the 23S rRNA, the elongation factor Tu, the RecA protein, and the β subunit of the RNA polymerase (*rpoB*)] has been suggested as an alternative to the 16S rRNA gene. The *rpoB* gene has recently been proposed as a target for PCR-DGGE analysis to follow LAB population dynamics during food fermentation (Rantsiou, et al. 2004). Although PCR-DGGE and PCR-TGGE are reliable, reproducible, rapid, and inexpensive (Muyzer 1999), their main limitation is that the community fingerprints they generate do not directly translate into taxonomic information – which is necessary to comparatively analyze sequences from excised and re-amplified DNA fragments to 16S rRNA gene sequences reported in nucleotide databases. More information about the identity of community members could be obtained by sequencing of PCR-DGGE/PCR-TGGE bands in the profiles and further comparison of the sequences with the available databases.

Single-strand conformation polymorphism (SSCP)-PCR analysis detects sequence variations between different DNA fragments, which are usually PCR-amplified from variable regions of the 16S rRNA gene. This technique is essentially based on the sequence-dependent differential intra-molecular folding of single strand DNA, which alters the migration speed of the molecules (Rolfs, et al. 1992). SSCP analysis requires uniform, low temperature, non-denaturing electrophoresis to maintain single-stranded DNA secondary structure. The discriminatory ability and reproducibility of SSCP-PCR analysis, which is generally most effective for fragment up to 400 bp in size, is also dependent on the position of the sequence variations in the gene studied (Vanechoutte 1996). SSCP-PCR analysis has been applied to evaluate diversity, succession, and activity of bacterial and yeast populations in raw milk Salers cheese (Duthoit, et al. 2003 and 2005; Callon, et al. 2006), and to characterize the surface flora of two French red-smear soft cheeses (Feurer, et al. 2004). However, similarly to PCR-DGGE/PCR-TGGE analyses, SSCP-PCR provides community fingerprints which can not be phylogenetically assigned.

An increasing number of new methodologies coupling PCR amplification with automated sequencing systems for laser detection of amplified, fluorescently labeled DNA fragments, has been recently proposed for DNA fingerprinting of microbial communities. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is a method that analyzes variation among 16S rRNA genes from different bacteria and gives information on microbial community structure (Osborn, et al. 2000). It is based on the restriction endonuclease digestion of fluorescent end-labeled PCR products. The individual terminal restriction fragments (T-RFs) are separated by gel (or capillary) electrophoresis and the fluorescence signal intensities

are quantified. Depending on the species composition of the microbial community, distinct profiles (T-RF patterns) are obtained as each fragment represents each species present. A relative quantitative distribution can be obtained, since the fluorescence intensity of each peak is proportional to the amount of genomic DNA present for each species in the mixture. Nevertheless, PCR bias could negatively affect the quantification of the real composition of the microbial community, as recently shown for a dairy-defined strain starter (Sánchez, et al. 2006).

Length heterogeneity-PCR (LH-PCR) is similar to T-RFLP. The difference between these two methods is that T-RFLP identifies PCR fragment length variations based on restriction site variability, whereas LH-PCR analysis distinguishes different organisms based on natural variations in the length of 16S rRNA gene (or other genes) sequences. In LH-PCR, a fluorescently labeled oligonucleotide is used as forward primer; it is coupled with an unlabeled reverse primer to amplify hypervariable regions of the 16S rRNA gene, which are located at the 5'-end of the bacterial gene. Labeled fragments are separated by gel (or capillary) electrophoresis and detected by laser-induced fluorescence with an automated gene sequencer. The relative amounts of amplified sequences originating from different microorganisms can be then determined. Because members of more than one taxonomic group can have LH-PCR products of the same size whereas, as stated above, T-RFLP analysis is likely to produce more fragments, the level of phylogenetic resolution of T-RFLP is higher than LH-PCR.

Use of T-RFLP and LH-PCR to profile microbial populations in fermented food is still limited. T-RFLP has recently been applied to perform semiquantitative analysis of metabolically active bacteria in dairy starters (Sánchez, et al. 2006) to assess microbial population dynamics during yogurt and hard cheese fermentation and ripening (Rademaker, et al. 2006), and to evaluate the surface microflora dynamics of bacterial smear-ripened Tilsit cheese (Rademaker, et al. 2005). LH-PCR has been applied to depict population structure and activity of the LAB community associated with Grana Padano cheese whey starters (Lazzi, et al. 2004; Fornasari, et al. 2006) and to monitor LAB succession during maize ensiling (Brusetti, et al. 2006).

The main limit of T-RFLP and LH-PCR is that with these techniques it is not possible to evaluate the population size within a microbial community. On the other hand, T-RFLP and LH-PCR share a number of advantages: a) efficiency, reliability, and high reproducibility; b) ability to provide the qualitative composition of different populations within relatively simple microbial communities, after evaluation of labeled fragments and c) ability to assess a direct phylogenetic affiliation of each member within the community. Relationships between the sizes of amplicons whatever obtained and gene phylogeny are predictable by comparison with previously published sequences of bacterial species, using web-based tools such as TAP (located at the RDP website; <http://rdp.cme.msu.edu/>) and T-Align (<http://inismor.ucd.ie/~talign/>). Similarly to PCR-DGGE/PCR-TGGE, T-RFLP and LH-PCR analyses could not be limited to ribosomal gene markers. The accumulating set of new sequences from various genes from less conserved DNA regions could allow the comparison of profiles for any gene system of interest. T-RFLP analysis of

mer and *amoA* genes has been applied to study bacterial communities in various environmental sites (Bruce 1997; Horz, et al. 2000).

Other PCR-based techniques have been proposed. In particular, fluorescence-labeled primer technology enabled to automate some applications of the most popular PCR-based techniques, such as PCR-Amplification Ribosomal DNA Restriction Analysis (PCR-ARDRA), rRNA gene Internal Spacer Analysis (RISA)-PCR, Arbitrarily Primed-PCR (AP-PCR), and Adaptor Fragment Length Polymorphism (AFLP), for phylogenetic and ecological studies of large sets of uncultured organisms from different habitats. Although in most cases the automation of these methods enhanced their sensitivity with respect to the classical approach, applications to food microbial communities in a culture-independent approach are still limited (Giraffa 2004).

RISA-PCR, also defined as 16S-23S rRNA gene Intergenic Transcribed Spacer (ITS)-PCR, is based on the amplification of the spacer region located between the 16S and the 23S rRNA genes. This region is extremely variable in size and sequence even within closely related taxonomic groups, and its amplification by PCR has been suggested as an excellent tool for strain characterization, typing, and for community fingerprinting (Nagpal, et al. 1998; Garcia-Martinez, et al. 1999). Whereas the applications of RISA/ITS-PCR as a strain typing tool will be reported later, here we show the potential of this technique as a culture-independent method. Following isolation of the total community DNA, PCR amplification of the 16S-23S intergenic spacer region is performed. The fragments are discriminated according to their length heterogeneity and their sizes compared to those of the GenBank database (Fisher and Triplett 1999). RISA/ITS-PCR offers interesting perspectives in examining particular taxonomic groups or species rather than the entire community. Indeed, several primers targeting different taxa on the same sample can be used to simultaneously evaluate the dynamics of each microbial group within a population. As an example, RISA/ITS-PCR has recently been applied to the microbial community analysis of Sausages (Ikeda, et al. 2005).

It should be mentioned that most of the reported studies have focused on the analysis of end products. DGGE and other techniques have, however, been more successfully applied in polyphasic studies to monitor the microbial dynamics of food ecosystems (Ercolini 2004; Ercolini, et al. 2004). By combining different methods (e.g. PCR-DGGE, cloning and sequencing of rRNA gene amplicons, and classical cultivation techniques) in a “polyphasic ecology” approach, it is now possible to profile time-dependent specific shifts in the composition of complex food microflora, evaluate and quantify non-cultivable food populations, and among these latter, to monitor the metabolically active microbial groups (Giraffa 2004).

3.2 In-situ Methods

Population fingerprinting techniques can successfully allow us to evaluate which organisms, in a given ecosystem, are present in a defined spatial element at a given

time, or to see how cells of both cultivable and non-cultivable bacterial species qualitatively evolve over both space and time. However, these techniques do not give exhaustive answers to more specific and urgent problems arising from the analysis of food-associated microbial communities. For example, how can we increase our knowledge of cell physiology, cell-to-cell interactions, and *in situ* modification of the microbial metabolism in natural ecosystems, especially in response to adverse environmental conditions? How can we quantify non-cultivable and/or non-dominant species/strains? Are there non-destructive methods of sample preparation to better evaluate spatial distribution and colonization of microorganisms in heterogeneous food matrices?

To answer to these questions, a number of *in situ* methods have been introduced (Amann, et al. 1995). The common trait of these methods is that morphologically intact cells (both cultivable and non-cultivable) can be identified and counted directly, e.g. in minimally disturbed samples. It is generally accepted that the term '*in situ* hybridization' (ISH) is restricted to whole-cell hybridizations in which viable cells are detected within their natural microhabitat. When organisms have been taken from a habitat or grown in laboratory media, the term 'whole cell' rather than '*in situ*' is preferred (Amann, et al. 1995; Vaid and Bishop 1999).

The fluorescence *in situ* hybridization (e.g. FISH) with rRNA targeted oligonucleotide probes has been developed over the last few years and, since its early application, a number of variants of the basic technique have been described until now. Ribosomal RNA represents a valid index of cell viability, as rRNA molecules are generally present in high numbers in viable cells. Microbial cells are first treated with appropriate chemical fixative, usually paraformaldehyde, and then immobilized onto microscopic slides, usually teflon coated. After facultative cell treatments to increase permeability to the probe, *in situ* hybridization with oligonucleotide probes is carried out. Generally, these probes are 15 to 20 nucleotide in length and are labeled covalently at the 5'-end with a fluorescent dye. After hybridization and stringent washing, specifically stained cells are observed by epifluorescence microscopy. A balance should be achieved in obtaining adequate permeability to allow the entry of reagents and the probe, without loss of cell morphology, while retaining the labeled probe within the cell.

FISH has made it possible to visualize the temporal and spatial distribution of microbes in aquatic, environmental and food ecosystems (Bouvier and del Giorgio 2003). FISH not only provides insight into microbial community structure, but can be combined with confocal laser scanning microscopy to depict the spatial arrangement of microbial communities within their habitat (Wagner, et al. 2003). FISH has been used to evaluate bacterial community structure and location in Stilton cheese (Ercolini, et al. 2003a, b), to detect *brevibacteria* on the surface of Gruyère cheese (Kolloffel, et al. 1999), to accurately enumerate *Pseudomonas* spp. in milk (Gunasekera, et al. 2003), and to determine cultivability and viability of probiotic bifidobacteria in fermented food (Lahtinen, et al. 2005). FISH has also been used to estimate the *in situ* activity of *Lactobacillus plantarum* in exponentially growing cells (de Vries, et al. 2004). A fundamental obstacle to the application of FISH in food is that the fragile structure of a fat-rich matrix (e.g. cheese) may impair the

maintaining of the spatial *in situ* distribution of microbial populations in foods. Recently, Ercolini, et al. (2003b) applied to Stilton cheese an embedding procedure using a plastic resin. The procedure obtained intact embedded cheese sections withstanding the hybridization reaction and represented a valid alternative to classical food cryo-sectioning.

Two recent improvements of the basic FISH procedure are the multiplex FISH and the multicolor FISH. The multiplex FISH essentially consists of independent multiple hybridizations with several probes carrying different fluorescence tags. The simultaneous investigation of complex biofilms composed of six bacterial species was possible by multiplex FISH analysis (Thurnheer, et al. 2004). In the multicolor FISH, species-specific probes are labeled with more than one fluorochrome in different ways, singly or in combination. Using this technique, seven *Bifidobacterium* spp. were differentially stained in mixed samples of cultured bacteria and feces from humans (Takada, et al. 2004).

The effectiveness of FISH is essential from both a phylogenetic and physiological point of view. Ineffective hybridization may result in an incomplete description of the community composition and wrong assumptions on the metabolic state of the cells (Bouvier and del Giorgio 2003). To this regard, FISH carries a number of drawbacks: (i) Variability related to methodological factors (target accessibility, type of fluorochrome, hybridization conditions) giving highly variable results; (ii) Variability related to the physiological cell state (e.g. slow-growing cells are difficult to detect because of the low rRNA content; damaged and/or stressed cells have variable rRNA content as well); (iii) Insufficient sensitivity to identify target sequences with low copy number. This latter limit led to the development of *in situ* PCR, a PCR method to amplify DNA within the cell. Compared to FISH, a labelling mix containing fluorescent nucleotides is deposited onto slides containing permeabilized and immobilized cells. After placing a microscope cover slip over the labelling mix, amplification is carried out. After PCR and washing steps, the slides are observed by epifluorescent microscopy. Dedicated *in situ* apparatus and kits were made commercially available to speed the overall procedure. *In situ* PCR could allow the analysis of communities of bacteria within their micro-environment or the identification of bacteria, particularly for slow-growing or uncultivable pathogenic strains, in clinical samples (Vaid and Bishop 1999). Nevertheless, no significant applications of *in situ* PCR to food microbiology are reported by literature.

3.3 Other Methods (Miscellaneous)

Flow cytometry (FCM) is a rapid and sensitive technique that measures each cell individually. Fluorescent stains are used with FCM to detect cells and to analyze population heterogeneity. The principle of the technique is based on the sorting of the stained cells through a process called hydrodynamic focusing in a narrow stream, where they follow each other one by one. The cells are then hit with a laser beam and, subsequently, the scattered light as well as induced fluorescence are

detected by several photomultipliers. FCM can be combined with whole-cell (or *in situ*) hybridization with fluorescently labeled rRNA-targeted oligonucleotide probes for a high-resolution automated analysis and selective enumeration of mixed microbial populations (Amann, et al. 1990). Moreover, a number of viability and metabolic activity probes are now available to also analyze physiological cell state and characteristics, such as membrane integrity, enzyme activities, and antibiotic susceptibility (Bunthof, et al. 2001; Bunthof and Abee 2002).

FCM-based methods have been applied to detect wild yeasts in breweries (Jespersen, et al. 1993), to analyze, in different proportions, subpopulations of variably stressed (or damaged) bacteria in probiotic products and dairy starters (Bunthof and Abee 2002), to determine the viability of probiotic strains during storage (Lahtinen, et al. 2006), and to improve LAB enumeration in mesophilic dairy starter cultures (Friedrich and Lenke 2006). As stated above, FCM is very sensitive. By FCM very rare cells, e.g. as rare as one per million, can be detected (Gross, et al. 1993). Moreover, the analysis can be further improved and actually transformed into a sort of preparative rather than merely analytical technique with an attached cell sorter device, which will specifically separate target cells. The capacity of the technique to sort individual cells is a powerful tool to face infraspecies (e.g. strain level) ecological studies. Cell sorting allowed the rapid selection and isolation from a strain of *Streptococcus thermophilus* of subpopulations of double mutants displaying phage resistance and good acid production (Viscardi, et al. 2003), and the concomitant assessment of viable, injured, and dead bifidobacteria cell subpopulations during bile salt stress (Ben Amor, et al. 2002).

A very effective method coupling PCR with dot-blot hybridization has also been developed. The method, defined as “reverse dot-blot hybridization,” is essentially based on the following principle: the target DNA of interest (generally, the rRNA gene) is amplified by PCR and labeled, and the labeled products are hybridized to an array of immobilized diagnostic probes. By using the simultaneous application of comprehensive sets of 16S and 23S rRNA-targeted, species-specific oligonucleotide probes, the direct detection of typical starter organisms without any preceding enrichment or cultivation steps could be obtained. It is now even possible to identify various LAB in fermented food at the species level within one working day (Ehrmann, et al. 1994; Schleifer, et al. 1995). More performing is the “quantitative hybridization approach,” which can be used to evaluate the abundance of the active microbial populations in fermented food. This method is based on total RNA extracted from food, which is denatured, slot blotted onto membrane and hybridized with chemiluminescence-labeled probes of the microbial groups to be monitored. Therefore, bound probes are quantified by densitometry relative to reference standards after autoradiography. The advantage is that, compared to PCR-based protocols, quantitative hybridization enables typical PCR biases to be avoided. By this method, culture-independent quantification of physiologically active microbial groups in lactic fermented maize dough was obtained (Ampe, et al. 1999b). Doubtless, the modern microarray, or DNA chip technologies, will open new horizons on the application of hybridization techniques. The DNA array technology will be described later.

4 Culture-dependent Techniques

Detection and identification of food isolates have, until recently, been performed mainly through biochemical and phenotypic methods. Nevertheless, taxonomists are aware that the phenotype may not accurately reflect true bacterial relationships. Phenotypic methods are generally labor intensive, time-consuming and do not always give unequivocal results. In addition, traditional methods are often insufficient to reliably identify many bacterial species and to monitor growth and dynamics of specific species and/or strains in complex bacterial communities. The most commonly used typing techniques are summarized in Table 1.2.

4.1 *Microbial Identification*

In many cases, assigning a name to bacterial isolates can be a difficult task. A wide range of bacterial species, including those that cause concern to the food industry (e.g. pathogenic bacteria), may pose serious problems in terms of identification. This has led to development of molecular identification methods, especially those based on PCR. The automation of many techniques, coupled with development of statistics and bioinformatics for microbiology, have led to a modification or replacement of conventional procedures in food microbiology laboratories.

4.1.1 DNA-DNA Hybridization Methods

The use of DNA probes for genes coding for rRNA offers a great potential in microbial identification. As rRNA (or other gene) sequences have become increasingly available, comparisons have revealed oligonucleotide stretches which are specific for different microbial taxa. These oligonucleotides can be labeled and used as probes in hybridization experiments with DNA of unknown isolates. Currently, oligonucleotide probes for the identification of almost all food-associated LAB are available (Schleifer, et al. 1995). A very useful tool is probeBase - an online resource for rRNA-targeted oligonucleotide probes (Loy, et al. 2003). The site (www.microbial-ecology.net/probebase) contains all the necessary information for probe sequences and protocols (even for FISH applications), as well as references concerning development and applications of the taxa-specific probes.

Different formats can be used for probe assays. For the dot-blot assay, the target nucleic acid has to be extracted from the cell and immobilized on a membrane. Then, either radioactively or non-radioactively labeled probes can be used for hybridization with the immobilized nucleic acid. The introduction of non-radioactive labeling methods (e.g. those based on chemiluminescence) has greatly facilitated the application of probes in food microbiology. A variation of this approach is the use of colony hybridization using group-specific probes. The advantage of this

Table 1.2 Advantages and Limitations of Using the Principal Molecular Techniques for Microbial Identification and Typing

	Advantages	Limits
A. Identification		
- DNA-DNA hybridization (e.g. rRNA-targeted oligo-nucleotide probes; dot-blot; colony hybridization, etc.)	High discrimination level; easy interpretation; high reproducibility	Cumbersome; time-consuming; expensive
- PCR-based methods (e.g. ARDRA-PCR, ITS (or RISA)-PCR; metabolic genes amplification)	Rapidity; high reproducibility; easy to use and interpret; low or moderate costs	Moderate discrimination level (to be raised by amplicon restriction, e.g. by ARDRA-PCR)
- DNA sequencing	High discrimination level; best accuracy and reproducibility; automated platforms available; public databases available	High technical competence needed; very expensive
B. Typing		
RFLP Methods		
- Ribotyping	High discrimination level; easy interpretation; high reproducibility; automated platforms available	Cumbersome; time-consuming; expensive
- REA-PFGE	Excellent discrimination level; excellent reproducibility; easy interpretation; public databases available	Cumbersome; difficult to use; long time to get result; moderate to high costs
PCR-based Methods		
- RAPD-PCR; Rep-PCR	High discrimination level; rapidity; easy use and interpretation; low costs	Moderate reproducibility; no public databases available
- ITS (or RISA)-PCR	Rapidity; easy use and interpretation; high reproducibility; low costs	Moderate discrimination level; no public databases available
- AFLP	Moderately easy to use and interpret; high discrimination power; high reproducibility; automated platforms available	High costs; no public databases available

Acronyms legend: ARDRA-PCR, Amplification Ribosomal DNA Restriction Analysis-PCR; ITS-PCR, Internal Transcribed Spacer-PCR; RISA-PCR, rRNA gene Internal Spacer Analysis-PCR; RFLP, Restriction Fragment Length Polymorphism; REA-PFGE, Restriction Endonuclease Analysis-Pulsed Field Gel Electrophoresis; RAPD-PCR, Randomly Amplified Polymorphic DNA-PCR; Rep-PCR, Repetitive element sequence-based-PCR; AFLP, Adaptor Fragment Length Polymorphism.

technique is that it allows the specific differentiation and quantification of target population(s) without the need of colony isolation and subculturing. In colony hybridization, bacteria are plated on membranes layered on appropriate agar media and allowed to form colonies. After lysis of the colonies, hybridization with a

labeled probe will show which and how many of the colonies contain the target sequence. The most recent development of colony hybridization is the *in situ* detection and identification (or ISH and its variant FISH) of whole cells with fluorescently labeled nucleotides.

Ribosomal RNA-targeted oligonucleotides have been used for the specific identification of LAB and yeasts (Schleifer, et al. 1995; Ampe, et al. 1999a). The use of DNA probes in colony or dot-blot hybridization experiments allowed the LAB community to be controlled in wine at different stages of wine-making, and to monitor the evolution of thermophilic lactobacilli belonging to *Lactobacillus helveticus*, *Lb. delbrueckii*, and *Lb. fermentum* during the early phases of Grana Padano cheese-making (Lonvaud-Funel, et al. 1991; Sohler and Lonvaud-Funel 1998; Giraffa, et al. 1998). Comparison of the use of rRNA probes and conventional methods also enabled identification of strains of *Lb. sakei* and *Lb. curvatus* isolated from meat (Nissen and Dainty 1995). Erlandson and Batt (1997) described a method using hydrophobic grid membrane filter colony hybridization for quantitative strain-specific detection of lactococci in bacterial populations. More recently, dot-blot hybridization has been applied to detect yogurt LAB in total fecal DNA (del Campo, et al. 2005). Colony hybridization has been applied for LAB identification (Betzl, et al. 1990; Hertel, et al. 1991), to characterize the microflora of Fontina cheese (Senini, et al. 1997), and to search for the presence of virulence genes related to diarrheal pathogenesis in *Escherichia coli* strains isolated from Pozol, an acid-fermented maize beverage consumed in Mexico (Sainz, et al. 2001).

Although species identification can be obtained with a high level of discrimination and reproducibility, hybridization techniques are being abandoned for taxonomic purposes. These methods are not particularly suited to the laboratory environment because protocols are generally cumbersome, time-consuming and expensive. A big obstacle is that multiple hybridizations for simultaneous identifications of more than one species are not possible. Another problem can be mis-identification, which may result from the presence of identical probe target sequences in phylogenetically diverse organisms. This has led to the development of commercial kits for specific microbial groups (e.g. food-associated pathogenic species), which result in the significant reduction of costs for those laboratories performing such tests. Another recent improvement has been the introduction of the “multiple probe concept,” which is based upon the assumption that the problem of mis-identification can be reduced by the simultaneous application of multiple probes targeting independent sites (Behr, et al. 2000).

4.1.2 PCR-based Methods

The on-line availability of DNA sequences of ribosomal RNA (rRNA) genes and rRNA gene spacers of practically all the known microbial species, and the presence of taxa-specific oligonucleotide stretches within the ribosomal locus has enabled these genes (or portions of them) to be routinely PCR-amplified and examined for differences indicative of genus and species identity. The rRNA gene sequences of

the target taxa are aligned with the sequences of phylogenetically associated organisms and, on the basis of the presence of both conserved and variable regions within the ribosomal (or other well-conserved) genes, genus- or species-specific oligonucleotide primers are designed. Amplified products, which can range from the single ribosomal genes to part or all the ribosomal locus, can be obtained by either simplex or multiplex PCR. A variant of this approach is ITS (or RISA)-PCR, whose principle has been explained above. Using primers published by Jensen, et al. (1993), several authors showed that ITS-PCR between the 16S and the 23S rRNA genes can produce amplicon profiles which are characteristics for each bacterial species when examined with high resolution non-denaturing acrylamide-bisacrylamide gel electrophoresis. Similar approaches have been applied for yeast identification (Arroyo-López, et al. 2006). Amplified products are then examined as a whole or subjected to restriction endonuclease analysis (such as in the case of amplified ribosomal DNA restriction analysis, ARDRA), which evidences RFLP of amplified genes within and between taxa and allows increasing the taxonomic resolution.

Several PCR amplification protocols are presently available for practically all food-associated *Lactobacillus* spp. (Giraffa and Neviani 2000), *Leuconostoc* spp. (Ward, et al. 1995; Moschetti, et al. 2000), and *Pediococcus* spp. (Mora, et al. 1997). Recently, the amplification by PCR of the intergenic spacer region (IGS) of rRNA gene followed by restriction RFLP analysis was evaluated as a potential method for distinguishing the 16 species belonging to the genus *Debaryomyces* (Quiròs, et al. 2006). Moreover, the increasing availability of non-ribosomal (metabolic) gene sequences has further revolutionized the PCR-based diagnostics. For example, the *pepIP* and *lacZ* genes can be respectively used to distinguish *Lb. delbrueckii* subsp. *lactis* from *Lb. delbrueckii* subsp. *bulgaricus* (Torriani, et al. 1999) and to identify *St. thermophilus* (Lick, et al. 1996). Similarly, *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* can be distinguished on the basis of primers designed on the histidine biosynthesis operon (Corroler, et al. 1998). Fortina, et al. (2001) described a multiplex PCR based on *pepC*, *pepN* and *htrA* targeted primers to identify *Lb. helveticus*. Jackson, et al. (2004) optimized a genus and species-specific multiplex PCR based on the *sodA* gene for identification of enterococci. Clearly, the use of non-ribosomal genes for taxonomic purposes is opening new possibilities to study the ecological evolution of microorganisms on the basis of the polymorphism of metabolic genes.

4.1.3 DNA Sequencing

DNA sequencing is considered the gold standard for microbial identification. The introduction in the early '90s of automated DNA sequencing machines and the development of bioinformatics (see later) have allowed individual laboratories to increase their output of DNA sequences from a few thousand base pairs per week to millions of base pairs per week, with much less effort and greater accuracy and reproducibility. DNA sequencing generally begins with PCR amplification of DNA (or RNA) directed at genetic regions of interest, followed by sequencing reactions,

which can be performed either by use of DNA sequencing or capillary gels of the amplified products. During electrophoresis, these fluorescently-labeled products are excited by an argon laser and are automatically detected. The resulting data are stored in digital form for subsequent processing into the final sequence with the aid of specialized software.

A number of sequence-based identification systems have been used to analyze the rRNA operon genes as well as other conserved genes in bacteria. Concerning the rRNA operon, the 16S rRNA gene is usually amplified for bacterial identification, whereas the 26S rRNA gene is generally used for yeast identification. Once the whole gene sequence is determined, it is compared to sequences from known microorganisms by the aid of specialized software programs and/or on-line tools. The programs use powerful algorithms to construct a phylogenetic tree (dendrogram) of how closely the sequences match and, hence, how closely the microorganisms are related. The accumulating set of information on rRNA sequences has proved to be effective for comparative identification of microorganisms, leading to recognition of thousands of microbial species. However, with regard to food-associated bacteria, non-ribosomal genes such as the *recA* gene (Felis, et al. 2001; Torriani, et al. 2001b) and the *rpmB* gene (Rantsiou, et al. 2004; Renouf, et al. 2006), are increasingly being used as phylogenetic markers for taxonomic purposes.

On the other hand, DNA sequencing is generally expensive and requires a high degree of technical competence to perform. Furthermore, sequencing all of the rRNA genes is not a practical method for routine microbial identification. This stimulated the sequencing of the hypervariable region in the 5'-end of the 16S rRNA gene (approx 500bp), which is sufficient for specific identification of most bacterial species (Patel, et al. 2000). Finally, automated DNA sequencers are still very expensive, with some costing in excess of \$100,000 (Olive and Bean 1999). To meet the increasing needs of the food industry, a number of private companies or associations (e.g. Belgian Coordinated Collections of Microorganisms [BCCM-LMG], Campden and Chorleywood Food Research Association [CCFRA]) provide a service to reliably and definitively characterize and identify bacterial isolates in a few hours at reduced costs (Dawson 2001).

4.2 Microbial Typing

Genotypic methods, based on molecular techniques, which are powerful to fingerprint specific DNA patterns that are characteristics for a single strain, form the mainstay of strain typing of LAB. The introduction of molecular biology techniques has yielded a variety of DNA-based typing methods, which can even discriminate between isolates of a given species. Depending on the technical aspects, genetic typing methods currently used can be divided into different categories with different taxonomic resolution: restriction fragment length polymorphism (RFLP) analysis of genomic DNA, PCR-based technologies and a miscellaneous of other methods, such as plasmid profiling or DNA sequencing. In the following sections, the most applied methods to type food-associated LAB will be discussed.

4.2.1 Methods Based on Restriction Fragment Length Polymorphism (RFLP) of DNA

Restriction fragment length polymorphism (RFLP)-based methods of total chromosomal DNA were among the first of the DNA-based typing schemes. Restriction endonuclease analysis (REA) includes whole-genome DNA extraction, its digestion with restriction endonucleases and separation of the resulting array of DNA fragments by gel electrophoresis. Using REA, discrimination down to strain level can be reached, although the high number of fragments makes the interpretation of the profiles difficult. However, over time, many RFLP-based approaches, such as ribosomal RNA gene restriction analysis (ribotyping) and REA-pulsed field gel electrophoresis (REA-PFGE), have been introduced to reduce the number of DNA fragments that are analyzed.

Ribotyping utilizes the similarities and differences found in rRNA genes. These genes are highly conserved, yet vary in number, size, and position within the same chromosome. After digestion and electrophoretic separation of whole chromosomal DNA, the separated DNA fragments are transferred to a membrane, fixed, and hybridized with a chemiluminescent rRNA gene probe. The resulting pattern of bands makes it possible to delineate species and strains on the basis of the difference in the RFLPs of ribosomal genes. Various species and individual strains of lactobacilli can be discriminated by ribotyping (Giraffa and Neviani 2000; Domig, et al. 2003). REA-PFGE, which is considered to be the gold standard among molecular typing methods, allows the comparison of 15 to 20 restriction DNA fragments generated after digestion of the whole chromosome by rare cutting restriction endonucleases. Bands are then separated by gel-electrophoresis under conditions that allow efficient resolution of high molecular size DNA fragments. REA-PFGE has been successfully used in the identification and subtyping of food-associated LAB and enterococci (Klein, et al. 1998; Giraffa and Neviani 2000; Domig, et al. 2003; Coppola, et al. 2006).

Both ribotyping and REA-PFGE are reproducible, easy to interpret, and highly discriminative; on the other hand, both techniques are difficult to apply in industry because they are cumbersome, difficult to use, and expensive (Olive and Bean 1999). This has led to an automation of methods. For example, the RiboPrinter system, which operates a completely automated ribotyping procedure, allows detection of the resulting hybridization pattern of bands on the membrane by a camera. The image is then transferred to a computer for analysis and is compared with a database containing 10,000 fingerprints of known bacteria. Using this system, which shows a high rate of inter-laboratory reproducibility, a bacterial isolate can be identified within eight hours (Dawson 2001). Strains of *E. faecium* resistant to vancomycin were successfully characterized by automated riboprinting (Brisse, et al. 2002).

4.2.2 PCR-based Methods

PCR-based DNA fingerprinting methods using arbitrary primers, such as arbitrarily primed PCR (AP-PCR) and randomly amplified polymorphic DNA (RAPD)-PCR,

have been developed for studying genomic DNA polymorphisms. Among PCR-based typing techniques, RAPD-PCR is the most popular typing technique applied to food ecosystems. RAPD-PCR is based on the use of short random sequence primers, nine or 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures. If two RAPD-PCR primers anneal within a few kilobases of each other, a PCR product will result. As the number and location of sites vary for different strains, a pattern of band is then generated which, in theory, is characteristic of a given bacterial strain. In recent years, hundreds of articles reported the application of RAPD-PCR to identify the presence, succession, and persistence of microorganisms (both useful and pathogens) in both fermented food and industrial environments (Maukonen, et al. 2003; Carminati, et al. 2004; Giraffa 2004). The numerous applications of this technique to different foods (and the relative references) will be detailed in the specific chapters of this book.

RAPD-PCR typing can be done quickly, especially in cases where fingerprinting is carried out with DNA from single-colonies growing on an agar plate. Therefore, RAPD-PCR is best suited for studies where specific bacterial strains are sought among a large number of isolates. Due to the low stringency of the PCR amplification, variability of RAPD-PCR fingerprints can sometimes be observed. The use of more than one primer and/or annealing temperatures (with increasing stringency) may improve reproducibility, but make the technique more laborious. A higher reproducibility of RAPD-PCR can be more practically achieved by careful standardization of the experimental methodology and by more objective comparison of DNA fingerprinting data. The development of bioinformatics has enabled the implementation of fingerprint databases, thus improving the interpretation and elaboration of RAPD-PCR data (Rossetti and Giraffa 2005).

In the repetitive element sequence-based PCR (Rep-PCR), repetitive chromosomal elements, which are randomly distributed in bacterial genomes, are the target of the PCR amplification. In Rep-PCR, primers anneal to repetitive parts of the chromosome and amplification occurs when the distance between primer binding sites is short enough to enable this. In Rep-PCR, amplification yields DNA fragments of varying size, which are separated by agarose gel electrophoresis (Versalovic, et al. 1991). Rep-PCR has been applied to characterize LAB isolated from fresh Sausages (Cocolin, et al. 2004).

ITS-PCR, which is a species-specific identification method, shows some potential for use as a microbial typing system, especially when applied to infraspecies identification of *E. gallinarum* and *E. faecium* (Domig, et al. 2003). A variation of this technique is PCR-ribotyping, which takes the advantage of the heterogeneity that exists within the spacer regions located between all the ribosomal genes. Improved discrimination, with respect to ITS-PCR, is obtained by using primers flanking conserved regions of 16S, 23S, and 5S rRNA genes, so that the intergenic spacer regions between the three ribosomal genes will be amplified. This technique has been used to type organisms such as *E. faecium*, *Escherichia coli*, *Enterobacter* spp., and *Listeria monocytogenes* (Domig, et al. 2003).

4.2.3 Miscellaneous

As stated above, the main criticism of the PCR-based typing systems (such as RAPD-PCR) is the limited interlaboratory reproducibility. To overcome this problem and to obtain a more sensitive discrimination between related strains, AP (or RAPD)-PCR could be performed using a fluorescence-labeled primer and the amplified fragments separated electrophoretically and detected by an automatic DNA sequencer (Cancilla, et al. 1992). Alternatively, more reproducible PCR-based microbial fingerprinting techniques (such as adaptor fragment length polymorphism or AFLP) could be used. AFLP involves restriction of total bacterial DNA with two endonucleases of different cutting frequencies, followed by ligation of the fragments to oligonucleotide adapters complementary to the sequences of the restriction site. Selective PCR amplification of the subset of fragments is achieved using primers corresponding to the contiguous sequences in the adapter and restriction site, plus a few nucleotides in the target DNA. Amplified fragments are then analyzed by gel electrophoresis. Unlike RAPD that uses multiple, arbitrarily chosen DNA regions to be amplified, the AFLP technique allows only two genomic regions to be amplified by selective primers and gives more reproducible results (Vos, et al. 1995; Janssen, et al. 1996). AFLP has recently been automated by using fluorescently dye-labeled primers, followed by separation of the labeled fragments through capillary electrophoresis under denaturing conditions and laser detection of the AFLP fragments using an automated analyzer (Gancheva, et al. 1999). A recent simplification of the AFLP method is the technique named SAU-PCR. Like the AFLP method, this technique uses primers based on the restriction enzyme recognition sequence, but it does not require the addition of linkers, and the products can be resolved on agarose gels. The proposed technique is based on the digestion of genomic DNA with the restriction endonuclease *Sau3AI* and subsequent amplification with primers whose core sequence is based on the *Sau3AI* recognition site (Corich, et al. 2005).

AFLP is a fairly new technique and, therefore, few data regarding its application to fingerprint food-associated microbes are available. AFLP proved a sensitive and reproducible technique for the typing of *Clostridium perfringens*, *Listeria monocytogenes*, and vancomycin-resistant *E. faecium* (Aarts, et al. 1999; Antonishyn, et al. 2000; McLauchlin, et al. 2000). The phenotypically closely related species *Lb. plantarum*, *Lb. pseudoplantarum*, and *Lb. pentosus* were discriminated on the basis of RAPD and AFLP patterns, which also allowed an effective infraspecific differentiation of 30 silage and cheese isolates to be obtained (Torriani, et al. 2001a).

5 Present Trends and Future Outlook

5.1 Quantitative PCR

It is well-known that standard PCR reactions are not quantitative. A promising tool for the advancement of studies on food-associated microbial populations, either cultivable or not cultivable, is the application of quantitative PCR (qPCR) to food

systems. Typical qPCR utilizes approaches originally developed in clinical microbiology, with the 5' fluorogenic exonuclease (TaqMan) assay representing the latest and widest applied development. By using an internal probe, which is labeled with fluorescent dyes, in addition to standard PCR amplification primers, TaqMan chemistry provides in-tube, real-time detection of PCR product accumulation during each amplification cycle and at very early stages in the amplification process. Using DNA as starting material, knowledge of the absolute composition, abundance, and structure of the microbial community, as well as the dynamics of individual populations, organisms or genes within that community can be obtained. Using RNA as a template and a real-time reverse transcription PCR assay, highly sensitive quantification of mRNA to measure levels of gene expression within a microbial population is possible.

Real-time PCR is increasingly applied for enumeration of bacteria, yeasts and molds in fermented foods such as fermented milk products (Bleve, et al. 2003; Furet, et al. 2004), dairy starters (Friedrich and Lenke 2006), and wine (Neeley, et al. 2005).

5.2 The Chip, DNA Array-based, Technology and its Applications

The DNA chip microarray technology is a direct result of the availability of genome sequence information. The technique involves very large (approximately 100,000) cDNA sequences or synthetic DNA oligomers being attached onto a glass slide (the chip) in known locations on a grid. An RNA sample is then labeled and hybridized to the grid and relative amounts of RNA bound to each square in the grid are measured. Such DNA chips can be used for simultaneous monitoring of levels of expression of all of the genes in a cell, in order to study whole genome expression patterns in various matrices during development. Moreover, since parallel hybridizations to hundreds or thousands of genes in a single experiment can be performed by high throughput DNA microarrays, direct profiling of microbial populations are achievable. Rudi, et al. (2002) combined the specificity obtained by enzymatic labeling of species-specific oligonucleotide probes with the possibility of detecting several targets simultaneously by DNA array hybridization with 16S rRNA gene from pure cultures. The hybridization of bulk DNA extracted from food to chip-bound probes is a promising tool for microbial community analyses in foods. In one recent development of this basic technique, Bae, et al. (2005) described genome-probing microarrays (GPM), which deposits hundreds of microbial genomes as labeled probes on a glass slide and hybridizes them with bulk community DNAs. GPM enabled quantitative, high-throughput monitoring of LAB community dynamics during fermentation of Kimchi, a traditional Korean food. Compared to currently used oligonucleotide microarrays, the specificity and sensitivity of GPM was remarkably increased (Bae, et al. 2005).

5.3 *Bioinformatics: An Essential Tool for Molecular Data Analysis*

Driven by automated DNA sequencing technology and the Human Genome Project, analysis of DNA and protein sequence data has spurred the dramatic growth of a new scientific discipline, bioinformatics, in the 1990s. Bioinformatics can be defined as the use of computers for the acquisition, management, and analysis of biological information. Bioinformatics combines *in silico* biological techniques with the DNA sequencing analysis approach. *In silico* biology combines statistical and mathematical algorithms with the need to manage and elaborate huge numbers of biological data. The development of bioinformatics has enabled improvement of the interpretation and elaboration of microbiological data. The acquisition of specialized, commercially available software packages – which are expensive and demand a high level of technical skill for their efficient use – is necessary so that the most important international microbial collections can manage, compare and implement databases holding information on nucleic acid (or protein) sequences, electrophoretic profiles, and phenotypic data.

One of the advantages of bioinformatics in relation to studying bacterial taxonomy and diversity concerns the possibility of sharing databases. As stated above, diagnostic tools based on RFLP or PCR have been developed for rapid inexpensive genotype assay. In addition, several microbial genomes have now been sequenced (for a review, see Klaenhammer, et al. 2005), while large numbers of DNA sequences have been compiled and are available via the World Wide Web. There is an urgent need to process this mass of information into a useful classification tool, which will require further automation and software development in order to effectively link different databases. In addition, bioinformatics could allow advances in functional genomics, e.g. conversion of the mass of sequence data presently available in public databanks into knowledge, so that microbial diversity could be assessed not only at the molecular level, but also at the functional level (Perego and Hoch 2001).

6 Final Comments

Modern food microbiologists are fortunate to have a variety of tools which provide very advanced molecular differentiation of microorganisms, and which can be tailored to fit the needs of both research laboratories and the food industry. Both cultivable and non-cultivable bacteria can be analyzed and microbial populations quantified, new microbial species can be isolated and characterized. Once efficiently integrated via advances in bioinformatics, molecular identification and fingerprinting techniques will provide more precise information on microbial taxonomy and functional diversity of a given food system at a particular time and space. Several of these molecular methods, once applied to the food industry, could

enable the creation of large reference libraries of typed organisms to which new strains can be compared either within the same laboratory or across different laboratories. Aspects such as changes in microbial populations, identification of contamination sources, management of customer/supplier disputes, assessment of sanitation programs, authentication of starter cultures, and verification of laboratory culture integrity could be then efficiently monitored. The choice of a molecular typing method will depend upon the needs, level of skill and resources of the laboratory concerned.

According to Forney, et al. (2004), some important limitations of these methods – especially those based on a culture-independent approach – should, however, be kept in mind. Most studies of microbial community diversity are based on extraction of total community DNA from a food sample; this step is often followed by a PCR amplification of a given gene target, which is generally a ribosomal gene. While this approach has proven to be very useful in understanding the microbial ecology of food, problems may arise at almost every step along the way, from the extraction of DNA to its amplification, up to the choice of the so-called “conserved” primers. Most importantly, since PCR amplification of DNA is a competitive enzymatic reaction, the small subunits rRNA templates in a sample are amplified according to their abundance. Populations that constitute less than 1 percent of the total community (which may still be present at levels higher than 10^5 cells/g or ml), generally go undetected in whatever generated amplification profiles. As a result, the actual community composition is difficult to determine (Forney, et al. 2004).

Another important weakness of culture-independent methods is that, in many cases, the taxonomic interpretation of data appears problematic. Most of these techniques are, in fact, based on the rRNA approach, e.g. nucleic acids which are directly retrieved from the sample and compared in a number of ways (e.g. sequence analysis, RFLP or length heterogeneity of the amplified pool of genes, etc.) to the rRNA sequence information of known bacteria present in the databases. But the “environmental sequences” rarely match the 16S rRNA of known bacteria (Amann 2000). As outlined above, we need, therefore, to expand our possibilities to investigate microbial diversity within natural populations by analyzing less conserved genes. Furthermore, culture-independent methods cannot completely avoid biases from estimating microbial diversity introduced by maceration and blending of the food sample, dilution of the homogenate, plating of dilutions onto agar media, and isolation and identification of colonies. Apart from *in situ* methods, determining community composition needs destructive sampling which, particularly in heterogeneous solid substrates (such as food), may result in alteration to the community, with little or no evidence that the isolated organisms cover all those present and active in the community (Giraffa 2004). The exciting review of Forney, et al. (2004) underlines pro and contra of molecular methodologies for studying microbial ecology, and suggests that our knowledge of microbial diversity is still very limited. At the present state of our knowledge, authors define microbial ecology very suggestively as “*The land of the one-eyed king*.”

References

- Aarts, H. J., Hakemulder, L. E., Hoef, A. M. van, 1999. Genomic typing of *Listeria monocytogenes* strains by automated laser fluorescence analysis of amplified fragment length polymorphism fingerprint pattern. *Int. J. Food Microbiol.* 49, 95–102.
- Amann, R., 2000. Who is out of there? Microbial aspects of biodiversity. *System. Appl. Microbiol.* 23, 1–8.
- Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., Stahl, D. A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919–1925.
- Amann, R. I., Ludwig, W., Schleifer, K. H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.
- Ampe, F., ben Omar, N., Guyot, J. P., 1999a. Culture-independent quantification of physiologically-active microbial groups in fermented foods using rRNA-targeted oligonucleotide probes: application to Pozol, a Mexican lactic acid fermented maize dough. *J. Appl. Microbiol.* 87, 131–140.
- Ampe, F., ben Omar, N., Moizan, C., Wachter, C., Guyot, J. P., 1999b. Polyphasic study of the spatial distribution of microorganisms in Mexican Pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl. Environ. Microbiol.* 65, 5464–5473.
- Antonishyn, N. A., McDonald, R. R., Chan, E. L., Horsman, G., Woodmansee, C. E., Falk, P. S., Mayhall, C. G., 2000. Evaluation of fluorescence-based amplified fragment length polymorphism analysis for molecular typing in hospital epidemiology: comparison with pulsed field gel electrophoresis for typing strains of vancomycin-resistant *Enterococcus faecium*. *J. Clin. Microbiol.* 38, 4058–4065.
- Arroyo-Lopez, F. N., Durán-Quintana, M. C., Ruiz-Barba, J. L., Querol, A., Garrido-Fernández, A., 2006. Use of molecular methods for the identification of yeasts associated with table olives. *Food Microbiol.* 23, 791–796.
- Bae, J. W., Rhee, S. K., Park, J. R., Chung, W. H., Nam, J. D., Lee, I., Kim, H., Park, Y. H., 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. *Appl. Environ. Microbiol.* 71, 8825–8835.
- Behr, T., Koob, C., Schedl, M., Mehlen, A., Meier, H., Knopp, D., Frahm, E., Obst, U., Schleifer, K. H., Niessner, R., Ludwig, W., 2000. A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. *System. Appl. Microbiol.* 23, 563–572.
- Ben Amor, K., Breeuwer, P., Verbaarschot, P., Rombouts, F. M., Akkermans, A. D. L., de Vos, W. M., Abee, T., 2002. Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Appl. Environ. Microbiol.* 68, 5209–5216.
- Betzl, D., Ludwig, W., Schleifer, K. H., 1990. Identification of lactococci and enterococci by colony hybridization with 23s rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 56, 2927–2929.
- Bleve, G., Rizzotti, L., Dellaglio, F., Torriani, S., 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. *Appl. Environ. Microbiol.* 69, 4116–4122.
- Bouvier, T., del Giorgio, P. A., 2003. Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): A quantitative review of published reports. *FEMS Microbiol. Ecol.* 44, 3–15.
- Brise, S., Fussing, V., Ridwan, B., Verhoef, J., Willems, R. J. L., 2002. Automated ribotyping of vancomycin-resistant *Enterococcus faecium* isolates. *J. Clin. Microbiol.* 40, 1977–1984.
- Bruce, K. D., 1997. Analysis of *mer* gene subclasses within bacterial communities in soils and sediments resolved by fluorescent-PCR-restriction fragment length polymorphism profiling. *Appl. Environ. Microbiol.* 63, 4914–4919.

- Bruseti, L., Borin, S., Mora, D., Rizzi, A., Raddadi, N., Sorlini, C., Daffonchio, D., 2006. Usefulness of length heterogeneity-PCR for monitoring lactic acid bacteria succession during maize ensiling. *FEMS Microbiol. Ecol.* 56, 154–164.
- Bunthof, C. J., Bloemen, K., Breeuwer, P., Rombouts, F. M., Abee, T., 2001. Flow cytometric assessment of viability of lactic acid bacteria. *Appl. Environ. Microbiol.* 67, 2326–2335.
- Bunthof, C. J., Abee, T., 2002. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. *Appl. Environ. Microbiol.* 68, 2934–2942.
- Callon, C., Delbes, C., Duthoit, F., Montel, M. C., 2006. Application of SSCP-PCR fingerprint to profile the yeast community in raw milk Salers cheese. *System. Appl. Microbiol.* 29, 72–80.
- Cancilla, M. R., Powell, I. B., Hillier, A. J., Davidson, B. E., 1992. Rapid genomic fingerprinting of *Lactococcus lactis* strains by arbitrarily primed polymerase chain reaction with 32P and fluorescent labels. *Appl. Environ. Microbiol.* 58, 1772–1775.
- Carminati, D., Perrone, A., Giraffa, G., Neviani, E., Mucchetti, G., 2004. Characterization of *Listeria monocytogenes* strains isolated from Gorgonzola cheese rinds. *Food Microbiol.* 21, 801–807.
- Cocolin, L., Rantsiou, K., Iacumin L., Urso, R., Cantoni, C., Comi, G., 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. *Appl. Environ. Microbiol.* 70, 1883–1894.
- Coppola, S., Fusco, V., Andolfi, R., Aponte, M., Blaiotta, G., Ercolini, D., Moschetti, G., 2006. Evaluation of microbial diversity during the manufacture of Fior di Latte di Agerola, a traditional raw milk pasta-filata cheese of the Naples area. *J. Dairy Res.* 73, 264–272.
- Corich, V., Mattiazzi, A., Soldati, E., Carraro, A., Giacomini, A., 2005. Sau-PCR, a novel amplification technique for genetic fingerprinting of microorganisms. *Appl. Environ. Microbiol.* 71, 6401–6406.
- Corroler, D., Mangin, I., Desmasures, N., Gueguen, M., 1998. An ecological study of lactococci isolated from raw milk in the Camembert cheese registered designation of origin area. *Appl. Environ. Microbiol.* 64, 4729–4735.
- Dawson, D., 2001. Molecular typing and identification of bacterial isolates. *New Food* 4, 63–65.
- del Campo, R., Bravo, D., Cantón, R., Ruiz-Garbajosa, P., García-Albiach, R., Montesi-Libois, A., Yuste, F. J., Abairra, V., Baquero, F., 2005. Scarce evidence of yogurt lactic acid bacteria in human feces after daily yogurt consumption by healthy volunteers. *Appl. Environ. Microbiol.* 71, 547–549.
- del Mar Lleó, M., Pierobon, S., Tafi, M. C., Signoretto, C., Canepari, P., 2000. mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Appl. Environ. Microbiol.* 66, 4564–4567.
- de Vries, M. C., Vaughan, E. E., Kleerebezem, M., de Vos, W. M., 2004. Optimizing single cell activity assessment of *Lactobacillus plantarum* by fluorescent *in situ* hybridization as affected by growth. *J. Microbiol. Meth.* 59, 109–115.
- Domig, K. J., Mayer, H. K., Kneifel, W., 2003. Methods used for the isolation, enumeration, characterization and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. *Int. J. Food Microbiol.* 88, 165–188.
- Duthoit, F., Godon, J. J., Montel, M. C., 2003. Bacterial community dynamics during production of registered designation of origin Salers cheese as evaluated by 16S rRNA gene single-strand conformation polymorphism analysis. *Appl. Environ. Microbiol.* 69, 3840–3848.
- Duthoit, F., Callon, C., Tessier, L., Montel, M. C., 2005. Relationships between sensorial characteristics and microbial dynamics in ‘Registered Designation of Origin’ Salers cheese. *Int. J. Food Microbiol.* 103, 259–270.
- Ehrmann, M., Ludwig, W., Schleifer, K. H., 1994. Reverse dot-blot hybridization: a useful method for the direct identification of lactic acid bacteria in fermented food. *FEMS Microbiol. Lett.* 117, 143–150.

- Ercolini, D., Hill, P. J., Hodd, C. E. R., 2003a. Bacterial community structure and location in Stilton cheese. *Appl. Environ. Microbiol.* 69, 3540–3548.
- Ercolini, D., Hill, P. J., Dodd, C. E. R., 2003b. Development of a fluorescence *in situ* hybridization method for cheese using a 16S rRNA probe. *J. Microbiol. Meth.* 52, 267–271.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Meth.* 56, 297–314.
- Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., Coppola, S., 2004. PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo mozzarella cheese. *J. Appl. Microbiol.* 96, 263–270.
- Erlandson, K., Batt, C. A., 1997. Strain-specific differentiation of lactococci in mixed starter culture populations using randomly amplified polymorphic DNA-derived probes. *Appl. Environ. Microbiol.* 63, 2702–2707.
- Felis, G. E., Dellaglio, F., Mizzi, L., Torriani, S., (2001). Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. *Int. J. Syst. Evol. Microbiol.* 51, 2113–2117.
- Felske, A., Akkermans, A. D., De Vos, W. M., 1998. Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription-PCR in temperature gradient gel electrophoresis fingerprints. *Appl. Environ. Microbiol.* 64, 4581–4587.
- Feurer, C., Vallaey, T., Corrieu, G., Irlinger, F., 2004. Does smearing inoculum reflect the bacterial composition of the smear at the end of the ripening of a French soft, red-smear cheese? *J. Dairy Sci.* 87, 3189–3197.
- Fisher, M. H., Triplett, E. W., 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 65, 4630–4636.
- Fleet, G. H., 1999. Microorganisms in food ecosystems. *Int. J. Food Microbiol.* 50, 101–117.
- Fontana, C., Vignolo, G., Cocconcelli, P. S., 2005. PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. *J. Microbiol. Meth.* 63, 254–263.
- Fornasari, M. E., Rossetti, L., Carminati, D., Giraffa, G., 2006. Cultivability of *Streptococcus thermophilus* in Grana Padano cheese whey starters. *FEMS Microbiol. Lett.* 257, 139–144.
- Forney, L. J., Zhou, X., Brown, C. J., 2004. Molecular microbial ecology: land of the one-eyed king. *Curr. Opin. Microbiol.* 7, 210–220.
- Fortina, M. G., Ricci, G., Mora, D., Parini, C., Manachini, P. L., 2001. Specific identification of *Lactobacillus helveticus* by PCR with pepC, pepN and htrA targeted primers. *FEMS Microbiol. Lett.* 198, 85–89.
- Friedrich, U., Lenke, J., 2006. Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence *in situ* hybridization. *Appl. Environ. Microbiol.* 72, 4163–4171.
- Furet, J. P., Quéneé, P., Tailliez, P., 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. *Int. J. Food Microbiol.* 97, 197–207.
- Gancheva, A., Pot, B., Vanhonacker, K., Hoste, B., Kersters, K., 1999. A polyphasic approach towards the identification of strains belonging to *Lactobacillus acidophilus* and related species. *System. Appl. Microbiol.* 22, 573–585.
- Garcia-Martinez, J., Acinas, S. G., Anton, I. A., Rodriguez-Valera, F., 1999. Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. *J. Microbiol. Meth.* 36, 55–64.
- Giraffa, G., 2004. Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol. Rev.* 28, 251–260.
- Giraffa, G., Rossetti, L., Muchetti, G., Addeo, F., Neviani, E., 1998. Influence of the temperature gradient on the growth of thermophilic lactobacilli used as natural starters in Grana cheese. *J. Dairy Sci.* 81, 31–36.
- Giraffa, G., Neviani, E., 2000. Molecular identification and characterisation of food-associated lactobacilli. *Int. J. Food Sci.* 12, 403–423.

- Giraffa, G., Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *Int. J. Food Microbiol.* 67, 19–34.
- Gram, L., Ravn, L., Rasch, M., Bruhn, J. B., Christensen, A. B., Givskov, M., 2002. Food spoilage – interactions between food spoilage bacteria. *Int. J. Food Microbiol.* 78, 79–97.
- Gross, H. J., Verwer, B., Houck, D., Recktenwald, D., 1993. Detection of rare cells at a frequency of one per million by flow cytometry. *Cytometry* 14, 519–526.
- Gunasekera, T. S., Dorsch, M. R., Slade, M. B., Veal, D. A., 2003. Specific detection of *Pseudomonas* spp. in milk by fluorescence *in situ* hybridization using ribosomal RNA directed probes. *J. Appl. Microbiol.* 94, 936–945.
- Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., Ishii, M., Igarashi, Y., 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *Int. J. Food Microbiol.* 109, 79–87.
- Hertel, C., Ludwig, W., Obst, M., Vogel, R. F., Hammes, W. P., Schleifer, K. H., 1991. 23S rRNA-targeted oligonucleotide probes for the rapid identification of meat lactobacilli. *Syst. Appl. Microbiol.* 14, 173–177.
- Horz, H. P., Rotthauwe, J. H., Lukow, T., Liesack, W., 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. *J. Microbiol. Meth.* 39, 197–204.
- Ikeda, S., Fujimura, T., Ytow, N., 2005. Potential application of ribosomal intergenic spacer analysis (RISA) to the microbial community analysis of agronomic products. *J. Agric. Food. Chem.* 53, 5604–5611.
- Jackson, C. R., Fedorka-Cray, P. J., Barrett, J. B., 2004. Use of a genus- and species-specific multiplex PCR for identification of enterococci. *J. Clin. Microbiol.* 42, 3558–3565.
- Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M., Kersters, K., 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiol.* 142, 1881–1893.
- Jensen, M. A., Webster, J. A., Straus, N., 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* 59, 945–952.
- Jespersen, L., Lassen, S., Jakobsen, M., 1993. Flow cytometric detection of wild yeasts in lager breweries. *Int. J. Food. Microbiol.* 17, 321–328.
- Klaenhammer, T. R., Barrangou, R., Buck, B. L., Azcarate-Peril, M. A., Altermann, E., 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol. Rev.* 29, 393–409.
- Klein, G., Pack, A., Bonaparte, C., Reuter, G., 1998. Taxonomy and physiology of probiotic lactic acid bacteria. *Int. J. Food Microbiol.* 41, 103–125.
- Kolloffel, B., Meile, L., Teuber, M., 1999. Analysis of *brevibacteria* on the surface of Gruyère cheese detected by *in situ* hybridization and by colony hybridization. *Lett. Appl. Microbiol.* 29, 317–322.
- Konaklieva, M. I., Plotkin, B. J., 2006. Chemical communication - Do we have a quorum? *Mini Rev. Med. Chem.* 6, 817–825.
- Lahtinen, S. J., Gueimonde, M., Ouwehand, A. C., Reinikainen, J. P., Salminen, S. J., 2005. Probiotic bacteria may become dormant during storage. *Appl. Environ. Microbiol.* 71, 1662–1663.
- Lahtinen, S. J., Ouwehand, A. C., Reinikainen, J. P., Korpela, J. M., Sandholm, J., Salminen, S. J., 2006. Intrinsic properties of so-called dormant probiotic bacteria, determined by flow cytometric viability assays. *Appl. Environ. Microbiol.* 72, 5132–5134.
- Lazzi, C., Rossetti, L., Zago, M., Neviani, E., Giraffa, G., 2004. Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. *J. Appl. Microbiol.* 96, 481–490.
- Lick, S., Keller, M., Bockelmann, W., Jochem Heller, K., 1996. Rapid identification of *Streptococcus thermophilus* by primer-specific PCR amplification based on its *lacZ* gene. *System. Appl. Microbiol.* 19, 74–77.

- Lonvaud-Funel, A., Fremaux, C., Bîteau, N., Joyeux, A., 1991. Speciation of lactic acid bacteria from wines by hybridization with DNA probes. *Food Microbiol.* 8, 215–222.
- Lopez, I., Ruiz-Larrea, F., Cocolin, L., Orr, E., Phister, T., Marshall, M., Van der Gheynst, J., Mills, D. A., 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Appl Environ Microbiol.* 69, 6801–6807.
- Loy, A., Horn, M., Wagner, M., 2003. probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucl. Acids Res.* 31, 514–516.
- Maukonen, J., Mättö, J., Wirtanen, G., Raaska, L., Mattila-Sandholm, T., Saarela, M., 2003. Methodologies for the characterization of microbes in industrial environments: a review. *J. Ind. Microbiol. Biotechnol.* 30, 327–356.
- McLauchlin, J., Ripabelli, G., Brett, M. M., Threlfall, E. J., 2000. Amplified fragment length polymorphism (AFLP) analysis of *Clostridium perfringens* for epidemiological typing. *Int. J. Food Microbiol.* 56, 21–28.
- Mora, D., Fortina, M. G., Parini, C., Manichini, P. L., 1997. Identification of *Pediococcus acidilactici* and *Pediococcus pentosaceus* based on 16S rRNA and *ldhD* gene-targeted multiplex PCR analysis. *FEMS Microbiol. Lett.* 151, 231–236.
- Moschetti, G., Blaiotta, G., Villani, F., Coppola, S., 2000. Specific detection of *Leuconostoc mesenteroides* subsp. *mesenteroides* with DNA primers identified by randomly amplified polymorphic DNA analysis. *Appl. Environ. Microbiol.* 66, 422–424.
- Muyzer, G., 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* 2, 317–322.
- Muyzer, G. A., de Waal, E. C., Uitterlinden, A. G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.
- Nagpal, M. L., Fox, K. F., Fox, A., 1998. Utility of 16S-23S rRNA spacer region methodology: how similar are interspace regions within a genome and between strains for closely related organisms? *J. Microbiol. Meth.* 33, 211–219.
- Neeley, E. T., Phister, T. G., Mills, D. A., 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. *Appl. Environ. Microbiol.* 71, 8954–8957.
- Nissen, H., Dainty, R., 1995. Comparison of the use of rRNA probes and conventional methods in identifying strains of *Lactobacillus sake* and *L. curvatus* isolated from meat. *Int. J. Food Microbiol.* 25, 311–315.
- Olive, D. M., Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. *J. Clin. Microbiol.* 37, 1661–1669.
- Osborn, A. M., Moore, E. R. B., Timmis, K. N., 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.* 2, 39–50.
- Patel, J. B., Leonard, D. G. B., Pan, X., Musser, J. M., Berman, R. E., Nachamkin, I., 2000. Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 16S rDNA bacterial identification system. *J. Clin. Microbiol.* 38, 246–251.
- Perego, M., Hoch, J. A., 2001. Functional genomics of Gram-positive microorganisms: review of the meeting, San Diego, California, 24 to 28 June 2001. *J. Bacteriol.* 183, 6973–6978.
- Quirós, M., Martorell, P., Valderrama, M., Querol, A., Peinado, J. M., de Silóniz, M., 2006. PCR-RFLP analysis of the IGS region of rDNA: a useful tool for the practical discrimination between species of the genus *Debaryomyces*. *Antonie van Leeuwenhoek* 90, 211–219.
- Rademaker, J. L. W., Peinhopf, M., Rijnen, L., Bockelmann, W., Noordman, W. H., 2005. The surface microflora dynamics of bacterial smear-ripened Tilsit cheese determined by T-RFLP DNA population fingerprint analysis. *Int. Dairy J.* 15, 785–794.
- Rademaker, J. L. W., Hoolwerf, J. D., Wagendorp, A. A., te Giffel, M. C., 2006. Assessment of microbial population dynamics during yogurt and hard cheese fermentation and ripening by DNA population fingerprinting. *Int. Dairy J.* 16, 457–466.
- Rantsiou, K., Cocolin, L., 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *Int. J. Food Microbiol.* 108, 255–267.

- Rantsiou, K., Comi, G., Cocolin, L., 2004. The *rpoB* gene as a target for PCR-DGGE analysis to follow lactic acid bacterial population dynamics during food fermentations. *Food Microbiol.* 21, 481–487.
- Renouf, V., Claisse, O., Lonvaud-Funel, A., 2006. *rpoB* gene: A target for identification of LAB cocci by PCR-DGGE and melting curves analysis in real-time PCR. *J. Microbiol. Meth.* 67, 162–170.
- Rolf, A., Schuller, I., Finckh, U., Weber-Rolf, I., 1992. Detection of single base changes using PCR. In: Rolf, A., Schuller, I., Finckh, U., and Weber-Rolf, I. (Eds.), *PCR: Clinical Diagnostics and Research*. Springer-Verlag, Berlin, pp. 149–167.
- Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *J. Microbiol. Meth.* 63, 135–144.
- Roszak, D. B., Colwell, R. P., 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* 51, 365–379.
- Rudi, K., Nogva, H. K., Moen, B., Nissen, H., Bredholt, S., Møretør, T., Naterstad, K., Holck, A., 2002. Development and application of new nucleic acid-based technologies for microbial community analyses in foods. *Int. J. Food. Microbiol.* 78, 171–180.
- Sainz, T., Wachter, C., Espinoza, J., Centurión, D., Navarro, A., Molina, J., Inzunza, A., Cravioto, A., Eslava, C., 2001. Survival and characterization of *Escherichia coli* strains in a typical Mexican acid-fermented food. *Int. J. Food Microbiol.* 71, 169–176.
- Sánchez, J. I., Rossetti, L., Martínez, B., Rodríguez, A., Giraffa, G., 2006. Application of reverse transcriptase PCR-based T-RFLP to perform semi-quantitative analysis of metabolically active bacteria in dairy fermentations. *J. Microbiol. Meth.* 65, 268–277.
- Schleifer, K. H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W., Amann, R., 1995. Application of molecular methods for the classification and identification of lactic acid bacteria. *Int. Dairy J.* 5, 1081–1094.
- Senini, L., Cappa, F., Cocconcini, P. S., 1997. Use of rRNA-targeted oligonucleotide probes for the characterization of the microflora from fermentation of Fontina cheese. *Food Microbiol.* 14, 469–476.
- Sohier, D., Lonvaud-Funel, A., 1998. Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiol.* 15, 391–397.
- Suzuki, M. T., Giovannoni, S. J., 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* 62, 625–630.
- Takada, T., Matsumoto, K., Nomato, K., 2004. Development of multi-color FISH method for analysis of seven *Bifidobacterium* species in human feces. *J. Microbiol. Meth.* 58, 413–421.
- Thurnheer, T., Gmur, R., Guggenheim, B., 2004. Multiplex FISH analysis of a six species bacterial biofilm. *J. Microbiol. Meth.* 56, 37–47.
- Torriani, S., Zapparoli, G., Dellaglio, F., 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. *Appl. Environ. Microbiol.* 65, 4351–4356.
- Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K., 2001a. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. *System. Appl. Microbiol.* 24, 554–560.
- Torriani, S., Felis, G. E., Dellaglio, F., 2001b. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Appl. Environ. Microbiol.* 67, 3450–3454.
- Vaid, A., Bishop, A. H., 1999. Amplification of fluorescently labeled DNA within Gram-positive and acid-fast bacteria. *J. Microbiol. Meth.* 38, 53–62.
- Vaneechoutte, M., 1996. DNA fingerprinting techniques for microorganisms. *Mol. Biotechnol.* 6, 115–142.
- Versalovic, J., Koeuth, T., Lupski, J. R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl. Acid Res.* 19, 6823–6831.
- Viscardi, M., Capparelli, R., Di Matteo, R., Carminati, D., Giraffa, G., Iannelli, D., 2003. Selection of bacteriophage-resistant mutants of *Streptococcus thermophilus*. *J. Microbiol. Meth.* 55, 109–119.

- Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T. van de, Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., 1995. AFLP: a new technique for DNA fingerprinting. *Nucl. Acid Res.* 23, 4407–4414.
- Walter, J., Tannock, J. W., Tilsala-Timisjarvi, A., Rodtong, S., Losch, D. M., Munro, K., Alatossava, T., 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol.* 66, 297–303.
- Wang, G. C. Y., Wang, Y., 1997. Frequency of formation of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from mixed bacterial genomes. *Appl. Environ. Microbiol.* 63, 4645–4650.
- Ward, L. J. H., Brown, J. C. S., Davey, G. P., 1995. Detection of dairy *Leuconostoc* strains using the polymerase chain reaction. *Lett. Appl. Microbiol.* 20, 204–208.
- Wintzingerode, F. V., Goebel, U. B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* 21, 213–229.

Chapter 2

Dairy Products

Salvatore Coppola, Giuseppe Blaiotta, and Danilo Ercolini

Abstract Approaches for studying microorganisms in food have undoubtedly changed. Advances in molecular biology have provided more information on food-associated bacteria, and have also provided the scientific community with sound, reliable and effective methods for detection, identification and typing of microorganisms from food. The main interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy productions and, possibly, to correlate the occurrence of certain microbial species and strains with desired flavor and sensorial traits of the products. Various molecular methods can be used depending on the level of information required by research. Microbiologists can be interested in identification, detection or typing of bacteria from a certain environment. Identification and detection can benefit from the availability of both culture-dependent and culture-independent techniques, whereas typing is an analysis performed on isolates and is, thus, strictly related to culture-dependent methods. The aim of this chapter is to describe how dairy microbiologists have made use of such advanced techniques to provide new insights in the study of the microbial ecology associated to dairy fermentation.

1 Diversity and Microbiological Aspects of Dairy Products

1.1 Introduction

Fermented dairy products are an important part of traditional diet, although their production/consumption is more common in some countries than others. This is clear from cheese databases (<http://www.indexmundi.com>) or from consumption data of fermented milks reported by Tamime and Robinson (1999).

Notoriously, they include a very wide variety of products obtained from milk by means of different combinations of fermentation and other biochemical activities with different technological interventions. Product diversity may be due to chemical

composition (mainly as moisture, fat and protein contents), texture, taste and aroma, as well as, quite typically, shape and size no less than final appearance. In the case of fermented milks, their diversity allows three great categories to be discriminated on the basis of the microbial activities typically occurring during their preparation: i) “Acidic,” such as Yogurt and Yakult; ii) “Acid-alcoholic,” such as Kefir and Koumys, and iii) “Viscous acid-alcoholic,” such as Scandinavian fermented milks.

Oberman and Libudzisz (1998) report an interesting classification of fermented milks into four types characterized by the microbial cultures used for their preparation. The first type gathers those produced using lactococci and leuconostocs, as is the case of Scandinavian fermented milks. The second, represented by Yakult, is produced using *Lactobacillus* strains. The third type is produced using cultures of thermophilic streptococci and lactobacilli – typically Yogurt. Finally the fourth type, comprising Kefir and Koumys, characterized by mixed microbial populations of lactic acid bacteria, yeasts, micrococci and acetic acid bacteria.

In contrast cheeses are usually classified according to criteria that rarely take account of microbial content and/or activities in each product: the milk species (goat, sheep, buffalo) is mentioned when milk other than from cow is used; according to their texture, cheeses are qualified as hard, semi-hard or soft. In some cases the time required for cheese making up to suitability for best consumption is taken into consideration, speaking of “fresh” or “unripened,” and “ripened” cheese; special categories are commonly recognized, such as “pasta filata cheese,” “blue-veined,” and “smeared.” Fox and McSweeney (2004) reported a long list of voluminous scientific literature, encyclopedias, pictorial books, country-specific or variety-specific books on cheese. Here it may be useful to recall that Ottogalli (2001) proposed an intriguing cheese classification by first distinguishing “*lacticinia*” (obtained from milk, buttermilk, cream or whey promoting protein clotting without the use of enzymes, but through biological acidification, addition of lactic or citric acid, or by means of combined action of acid with heat; and represented, as main products, by Ricotta cheese, Queso blanco, Mascarpone) from “*formatica*” (true cheeses, obtained after milk clotting with animal, plant or microbial rennet, followed by whey draining). The latter are further split into six classes. Different classification schemes for cheese were reported by McSweeney, et al. (2004), none of them considering the microbial diversity characterizing different types of cheese. Mucchetti and Neviani (2006) have recently listed cheeses according to the following categories: i) cheeses produced with pasteurized milk and selected starter; ii) cheeses produced with pasteurized milk and natural starter; iii) cheeses produced with thermal treated milk and natural starter; iv) cheeses produced with raw milk and selected starter; v) cheeses produced with raw milk and natural starter, and vi) cheeses produced with raw milk without starter addition.

Actually, as well pointed out by Johnson (1998), the diversity of cheese making processes makes cheese a complex subject microbiologically; according to Johnson (1998) it is a misconception to think of cheese microflora in terms of the type of cheese, for example, all cheddars, blue cheeses, and so on. He emphasizes the occurrence of adventitious, non-starter, non-deliberately added contaminants that

can really cause each individual cheese (not type) to have its own unique microflora. He also considers conventional methods (both those currently used to isolate microorganisms and those to differentiate the isolates by biochemical tests) as unsatisfactory for studying a dynamic ecological system such as cheese microflora, and applications of molecular techniques like PCR (with reference, of course, to the time he was writing), just useful to determine the presence of individual species or strain, offering the possibility to identify the proverbial “needle-in-a-haystack.”

Today we can assume that modern molecular methods of analysis within polyphasic approaches are available to obtain information about microorganisms occurring in the various dairy products; also with a view to discriminating species whose cells are viable at the moment of the analysis from those formerly present, but no longer active, due to one or more factors: technological stress, depletion of specific nutrients, microbial antagonism, modified adverse environmental conditions occurring during cheese manufacture and ripening. Beresford, et al. (2001) cited an interesting review by O’Sullivan (1999) to maintain that the development of culture-independent methods for microbial analysis has revolutionized microbial ecology. Advanced procedures have also been implemented to establish the location of specific microorganisms in particular parts of the product sample (Ercolini, et al. 2003a,b). A recent review by Spiegelman, et al. (2005) discussed the most powerful methods exploitable in environmental microbiology for the characterization of microbial consortia and communities. Zhang and Fang (2006) critically reviewed emerging techniques involving real-time polymerase chain reaction for quantification of microorganisms in environmental samples. Friedrich and Lenke (2006) applied multiplex quantitative Real-Time PCR (q-PCR) and flow cytometry-FISH to enumeration of lactic acid bacteria (LAB) in a mesophilic dairy starter culture. Various approaches to studying gene expression in complex environments are also available (Saleh-Lakha, et al. 2005). Significant applications showing technical aspects, potentials and limits of these methods will be discussed in this chapter to draw special attention to those that can be particularly useful in studying the microbial ecology of cheese.

The study of microbial ecology associated with dairy fermentation is fundamental to understand the bases of important traits of dairy products. Interestingly, microbiological aspects are not usually taken into account in cheese classification systems.

The microbiota of each dairy product (as well as, of course, each fermented food) has its own history, during which the microbial population structure changes under the influence of continuous shifts in environmental factors occurring during its preparation. Therefore, microorganisms, at species and strain level, must be monitored at least during the most effective technological phases, where it is important to have certain microbial activities in order to achieve the expected quality of the final product. Changes in the microbial community during the various phases of dairy production are particularly important for achieving a satisfactory description of the microflora occurring, especially in typical products obtained by traditional procedures. This could help in understanding the basis for specific sensorial traits and/or their seasonal variations. In the case of PDO (Protected Denomination of Origin) cheeses, it would be important to recognize an association between microbial

diversity and the area of production that may enhance the link between microbiota, the environment and sensorial quality of these traditional productions.

A satisfactory study of the microbiology of dairy fermentations must also examine all the important technological phases of production. Therefore, the significant steps of cheese production will be described below with particular attention to their possible influence on the microbiota of cheese.

1.2 Technological Production Phases of Dairy Products

Milk Pre-treatment and Standardization

Although most industrial dairy products are produced from pasteurized milk, a large number of raw milk cheeses are increasingly described as celebrated traditional on-farm-made cheeses and commercially proposed as gastronomic specialities, emphasizing their distinctive flavor and suggesting the best way to consume them. In the microbiological literature these types of cheese are attracting increasing coverage, showing once more the importance of the biodiversity of raw milk native microflora to achieve the roundest, most pleasant and palatable traits. Studies are needed on molecular methods for monitoring both useful and dangerous microorganisms during practices performed in raw milk cheese making, such as milk storage/ripening (usually at a low temperature) or milk skimming for reducing the fat content. Indeed, cold storage is known to cause physical and chemical changes to milk, and to be selective for the development of psychrotrophic microflora. Great interest is merited by the case of partial milk-skimming by natural creaming typically performed within the manufacture of famous Italian semi-fat hard and long-ripened cheeses (Parmigiano-Reggiano and Grana Padano), promoting fat floating to the surface of the raw milk contained in wide trays for as long as six to 12 hours. Within this singular and uncommon practice, fat droplets appearing on the surface lead to microbial enrichment of the cream and a reduction in the microbial content of the skimmed milk lying below (Mucchetti and Neviani 2006).

Starter Addition

For starter cultures, a particularly valid nomenclature is proposed by Limosowtin, et al. (1996), who define “Mixed Strain Starters” (MSS) as the cultures that include many species and strains in unknown proportions, and “Defined Strain Starters” (DSS) as those containing known quantities of known strains. Within the first category, the authors list “Artisanal or Natural Mixed Starter Cultures” (NMSS), “Thermophilic Mixed Starter Cultures” (TMSS) and “Mesophilic Mixed Starter Cultures” (MMSS); within the second (DSS) “Thermophilic Defined Starter Cultures” (TDSS) and “Mesophilic Defined Starter Cultures” (MDSS). It can be assumed that most DSS, as

well as many single strain starters, are commercial starters produced by specialized laboratories; MSS – mainly NMSS – are prepared and managed by the dairies, often following traditional procedures that, for the same type of cheese, can differ somewhat among the various manufacturers. Many cheeses are traditionally produced without starter addition nor by using back-slopping practices. Natural whey cultures are microbial cultures naturally occurring in the whey extracted in previous cheese making, stored at room temperature or variously handled, and then used in the manufacture of the following day with a back-slopping practice. Instead, natural artisanal milk cultures are generally obtained by incubating, at appropriate temperatures, a large amount of raw milk after mild heating to destroy undesirable microflora. Indeed, they commonly contain strains of thermophilic microorganisms, such as thermobacteria, thermophilic streptococci and enterococci. Moreover, the dairy industry can benefit from the use of other microbial cultures called “adjuncts;” they are not essential for the technological process in itself, but are selected and used with specific additional purposes. They can be “reinforcing cultures” to be used to accelerate or standardize acidification; “flavoring cultures” to enhance aroma production; “protective cultures” to inhibit pathogens or spoilage agents, or “health cultures” to enrich the product with probiotic strains.

The addition of starters and/or adjuncts to cheese milk causes an immediate change in the microbiota of the technological ecosystem concerned, as loads of at least 10^6 cells per ml of milk of each important microbial type are applied. Thus, the structure of the population under investigation is strongly – and often permanently – influenced after the inoculum.

Milk Clotting

Rennet-coagulated cheeses represent, by far, the greater part of solid dairy products. Liquid, powder or tablet rennets are usually special preparations with little to no significant microbial content. Among traditional cheeses a number of more or less long ripened products are manufactured by the use of a rough type of rennet prepared in the form of a paste from the fourth stomach (abomasum) of suckling goat kid or lambkin. These rennets, in addition to chymosin and other proteases, contain some lipases that, during cheese ripening, are responsible for reactions that produce a distinct piquant taste. Several studies have shown that these rennet pastes might have a microbial content, representing a special additional source of microorganisms for the cheese (P. Deiana, personal communication). Modern research on the microbial content of raw rennet is, to our knowledge, nonexistent and no information is available about the possible presence of stressed or unculturable microorganisms.

In any case, depending on the type of cheese, coagulation time is variable, generally predefined through the quantity and quality (clotting strength) of the rennet. Milk fat droplets, whey with water-soluble components and microbial cells are entrapped within the casein network, e.g. inside the pores among aggregates of micelles. Microbial growth may also occur, of course, in a good nutritional environment and with favorable temperatures, no longer as planktonic cells but growing as colonies

in a solid matrix. Sampling at this stage of cheese making may be of interest for chemical investigation. However, microbial populations at the end of the clotting process cannot yet be referred to cheese, due to the fact that part of the microorganisms entrapped in the coagulum will be eliminated from the system by subsequent technological phases.

Curd Cutting, Cooking and Draining

Once the coagulum firmness required for the specific cheese variety has been reached, the curd is cut with knives or wire-tools into small pieces. Cut size also strongly depends on the cheese type, as the firmer and larger the curd pieces, the higher the moisture content of the cheese. According to the Italian dairy tradition, curd piece size is typically named after fruits or seeds of similar size: walnut, little walnut, almond, hazelnut, pea, small pea and grain. Long ripening cheeses require curd cutting at the smallest size (grain); fresh or brief-ripening cheeses at the largest one (walnut). With cutting, caseins continue to interact and squeeze out the whey entrapped (with all its water-soluble components, lactose included) and some microbial cells as well (more cocci than rods). Curd pieces shrink, become firmer and, depending again on the cheese variety, they can be differently processed. Syneresis may be enhanced by lowering the pH (hence, counting on starter effectiveness), increasing the temperature and stirring the curd (performing, in this case, the process of curd cooking). Alternatively, the curd pieces can be promptly separated from the whey, drained and subjected to the subsequent technological phase of molding. However, curd treatments are always ecologically important, involving selective pressures with major effects upon microorganisms and their activities.

Molding

Molding can be ecologically important due to the possibility of contamination occurring during curd handling. It is generally recognized that chemical cleaning and sanitation of tanks, vats and other tools used within cheese making – made of proper modern materials – can reduce contamination considerably, whereas it is more difficult to achieve satisfactory results when the cheese is exposed to the work environment. This is considered the main source of adventitious cheese microflora, commonly including non-starter LAB, and is responsible for important activities during cheese ripening. Molding is, therefore, another stage of cheese making during which the cheese microflora can change.

Salt Addition

Salt is added in cheese making to improve its taste and to lower the water activity. In some cases it is added as a solid to milled curd; in others, cheeses removed from

the forms are brined, and in further cases the cheese molds are dry salted by rubbing or sprinkling salt on their surfaces.

Salting causes selective pressure upon microorganisms. Brines are often a source of cheese contamination by salt-tolerant microorganisms: yeasts such as *Debaryomyces hansenii* and its imperfect form *Candida famata*; bacteria such as staphylococci, micrococci, enterococci, corynebacteria and some LAB.

Ripening

It is well-known that cheese ripening occurs in a variety of environmental conditions, depending on cheese type, and often in natural or cellar conditions, where it is uncontrolled and difficult to reproduce. Moreover, this final process of cheese making is difficult to describe because it consists of a complex succession of events conditioned by the previous technological stages, with the contribution of secondary adventitious microflora, under the influence of the cheese storage environment and, in some cases, caused by curing practices. Then, further complication may be encountered in some cases, where ripening proceeds without heterogeneity within the same cheese mold: blue-veined cheeses include portions strongly affected by both growth and activity of *Penicillium roqueforti*; surface-ripened cheeses are characterized by centripetal ripening due to diffusion of enzymes produced by the surface microflora. Such complexity requires polyphasic analytical approaches including physical, chemiometric, molecular and cultural procedures to be performed on several samples from various parts of the same cheese mold.

1.3 Microbial Diversity in Dairy Products

For about one decade, studies of the microbial ecology of cheese have focused on explaining the relationship between microbial population succession (in terms of implantation/growth/colonization), enzyme production/activity (with reference to milk and rennet enzymes too, in addition to those of a microbial origin) and cheese texture, taste and flavor development. Great emphasis has been given to the importance of microbial diversity and the role of non-starter microorganisms. Nevertheless, there is scant information, both in terms of quality and quantity, to be used for total technological control/management of cheese quality, or for producing pasteurized milk cheese with flavor resembling raw milk cheese.

Therefore, given that molecular techniques can quantify both microbial species that can be encountered, targeting rRNA genes and their activity, and evaluating the expression of functional genes according to methods already applied to the study of natural environments (Saleh-Lakha, et al. 2005), it may be worth briefly recalling microorganisms and metabolic activities that are expected during the most important above-mentioned phases of cheese making.

In evaluating cheese milk quality, species- or biotype-specific DNA sequences can be targeted for detection of pathogenic microorganisms. Therefore, sequences of genes encoding for toxins can be used both to detect the producer's occurrence and ascertain the specific gene expression during milk cold-storage, ripening or partial skimming. During these pre-treatments, microbiological investigations should also deal with monitoring the effective growth of useful microflora in comparison with that of psychrotrophic flora regarded as spoilage agents. Of the latter, *Pseudomonas* spp., *Alteromonas putrefaciens*, *Alcaligenes faecalis*, *Arthrobacter globiformis*, *Serratia* spp., *Enterobacter* spp. and *Flavobacterium* spp. may be responsible for anti-technological activities: undesirable proteolytic and lipolytic activities, ammonia production, diacetyl mineralization and production of many off-flavors, like fruity, sweetish, fecal or putrid.

Within production of long-ripened cheese, the presence of the anaerobic spore-former *Clostridium tyrobutyricum* – considered the causative agent of late blowing spoilage – can be verified in cheese-milk by species-specific PCR according to Klijn, et al. (1995a) or by PCR-DGGE (Cocolin, et al. 2004).

In studying microbial ecology during cheese manufacture, microorganisms need to be monitored with reference to their taxonomic and metabolic diversity to be consistent with technological protocol. Of course, mixed and more complex microflora must be expected in raw milk cheeses and when natural starter cultures are used. In these cases, in fact, the acidification process relies on the indigenous microflora, usually taxonomically complex. Considering the temperature ranges suitable for the various LAB groups, both thermophilic and mesophilic species can occur. The most important species of the microflora occurring during the manufacture of the main types of cheese are reported in Table 2.1.

Only residual amounts of lactose are generally available at the end of cheese manufacture. Thus, the initially dominant starter microorganisms responsible for acidification and flavor production from lactose and citrate during the first phase of cheese making are destined to progressively decrease from the phase of cheese ripening. Thereafter, depending on the cheese variety, secondary and/or adventitious microflora begin their growth, carrying out their activities. The latter involve both metabolites formerly produced by starter bacteria and the other constituents of cheese curd, like proteins and fat, generally transformed to a lesser extent within the first stage of cheese making. Moreover, they complement biochemical activities arising from residual milk plasmin, rennet enzymes and starter cell autolysis. However, secondary and adventitious microflora should not be regarded as agents of single specific processes, but as contributors to the overall complex transformations influencing the final cheese quality.

Milk proteins and lipid metabolism by dairy bacteria, yeast and molds will release key flavor molecules that will characterize each kind of cheese. Such compounds are the result of the diversity of the microorganisms and their activities during cheese ripening.

Microbial ecology of cheese, as well as of other fermented dairy products, can greatly benefit from molecular tools supporting identification of microbial species and strains occurring during the production processes, discriminating and quantifying

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type

<i>Taxon</i>	Products
Starter Microorganisms (mainly responsible for production of lactic acid, ethanol, acetaldehyde and diacetyl)*	
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	Villi **: (1); Kefir (1); Cheddar (3); Edam-Gouda (4); Cottage cheese (6); Camembert (3); Brie (4); Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Artisanal Emmenthal (9); Pecorino Sardo (9); Majorero (9); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Toma Piemontese (29); Fiore Sardo (30); Stilton (31); Ragusano (32)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i>	Villi (1); Kefir (1); Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Water buffalo Mozzarella (8)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	Villi **: (1); Kefir (1); Cheddar (3); Edam-Gouda (4); Cottage cheese (6); Camembert (3); Brie (4); Blue-veined cheeses (3); Limburger and other surface-ripened cheeses (6); Water buffalo Mozzarella (8); Artisanal Emmenthal (9); Pecorino Sardo (9); Majorero (9); Canestrato Pugliese (22); Toma Piemontese (29)
<i>Streptococcus salivarius</i>	Salers (25)
<i>Streptococcus thermophilus</i>	Kefir (1); Yogurt (2); Gorgonzola (4); Brie (4); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Montasio (14); Provolone (14); Asiago (21); Scamorza Altamura (23); Toma Piemontese (29); Ragusano (32)
<i>Streptococcus macedonicus</i>	Kasseri (19); Asiago (20); Montasio (20); Scamorza Altamura (23); Salers (25); Toma Piemontese (29)
<i>Enterococcus faecium</i>	Artisanal Emmenthal (9); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (27); Toma Piemontese (29)
<i>Enterococcus faecalis</i>	Artisanal Emmenthal (9); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (27); Toma Piemontese (29); Ragusano (32)
<i>Enterococcus durans</i>	Scamorza Altamura (23); Fiore Sardo (27); Toma Piemontese (29)
<i>Leuconostoc lactis</i>	Water buffalo Mozzarella (8); Ragusano (32)
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i>	Artisanal Emmenthal (9); Artisanal Mozzarella (16); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Pecorino Sardo (9); Majorero (9); Salers (25); Ragusano (32)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
<i>Leuconostoc mesenteroides</i> subsp. <i>dextranicum</i>	Kefir (1); Water buffalo Mozzarella (8)
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	Villi (1); Kefir (1); Water buffalo Mozzarella (8)
<i>Leuconostoc pseudomesenteroides</i>	Salers (25);
<i>Leuconostoc argentinum</i>	Canestrato Pugliese (22); Ragusano (32)
<i>Leuconostoc gelidum</i>	Fior di Latte di Agerola (10)
<i>Weissella hellenica</i>	Artisanal Mozzarella (17)
<i>Weissella paramesenteroides</i>	Fior di Latte di Agerola (10)
<i>Weissella viridescens</i>	Scamorza Altamura (23)
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	Yogurt (2); Gorgonzola (4); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Caciocavallo Silano (18); Asiago (21); Ragusano (32)
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	Kefir (1); Emmenthal (4); Artisanal Emmenthal (9); Water buffalo Mozzarella (8); Grana Padano (12); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Parmigiano Reggiano (13); Caciocavallo Silano (18); Scamorza Altamura (23); Ragusano (32)
<i>Lactobacillus helveticus</i>	Kefir (1); Emmenthal (4); Artisanal Emmenthal (9); Mozzarella (4); Artisanal Mozzarella (17); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Grana Padano (12); Parmigiano Reggiano (13); Pecorino Romano (14); Pecorino Sardo (9); Majorero (9); Montasio (14); Asiago (14); Provolone (14); Scamorza Altamura (23)
<i>Lactobacillus casei</i>	Caciocavallo Silano (18); Canestrato Pugliese (22); Fiore Sardo (30); Ragusano (32)
<i>Lactobacillus casei</i> subsp. <i>casei</i>	Artisanal Mozzarella (17); Fior di Latte di Agerola (10)
<i>Lactobacillus casei</i> subsp. <i>pseudoplantarum</i>	Water buffalo Mozzarella (8)
<i>Lactobacillus casei</i> subsp. <i>alactosus</i>	Kefir (1)
<i>Lactobacillus casei</i> subsp. <i>rhamnosus</i>	Kefir (1)
<i>Lactobacillus casei</i> subsp. <i>paracasei</i>	Scamorza Altamura (23)
<i>Lactobacillus paracasei</i>	Caciocavallo Silano (18); Salers (25); Fiore Sardo (30)
<i>Lactobacillus paracasei</i> biovar <i>Shirota</i>	Yakult (2)
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	Fior di Latte di Agerola (10)
<i>Lactobacillus plantarum</i>	Artisanal Mozzarella (17); Water buffalo Mozzarella (8); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Canestrato Pugliese (22); Salers (25); Fiore Sardo (30); Ragusano (32)
<i>Lactobacillus kefir</i>	Kefir (1)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

<i>Taxon</i>	<i>Products</i>
<i>Lactobacillus brevis</i>	Kefir (1)
<i>Lactobacillus cellobiosus</i>	Kefir (1)
<i>Lactobacillus fermentum</i>	Artisanal Emmenthal (9); Grana Padano (12); Artisanal Mozzarella (17); Caciocavallo Silano (18); Scamorza Altamura(23); Ragusano (32)
<i>Kluyveromyces lactis</i>	Kefir (1)
<i>Kluyveromyces marxianus</i> subsp. <i>bulgaricus</i>	Kefir (1)
<i>Kluyveromyces marxianus</i> subsp. <i>marxianus</i>	Kefir (1)
<i>Saccharomyces florentinus</i>	Kefir (1)
<i>Saccharomyces globosus</i>	Kefir (1)
<i>Saccharomyces unisporus</i>	Kefir (1)
Co-starter or adjuncts (typical components of the secondary microflora of some products, often intentionally introduced)	
<i>Lactobacillus acidophilus</i>	Kefir (1); Bio-Yogurt (2)
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	Bio-Yogurt (2)
<i>Lactobacillus paracasei</i> biovar <i>Shirota</i>	Bio-Yogurt (2)
<i>Lactobacillus rhamnosus</i>	Bio-Yogurt (2)
<i>Lactobacillus reuteri</i>	Bio-Yogurt (2)
<i>Bifidobacterium</i> spp.	Bio-Yogurt (2)
<i>Enterococcus faecium</i>	Bio-Yogurt (2)
<i>Enterococcus faecalis</i>	Bio-Yogurt (2)
<i>Pedococcus acidilactici</i>	Bio-Yogurt (2)
<i>Penicillium camemberti</i>	Camembert (3); Brie (4)
<i>Penicillium roqueforti</i>	Roquefort and other blue-veined cheeses (3); Gorgonzola (4); Stilton (9); Danish blue (9); Stilton (31)
<i>Geotrichum candidum</i>	Viili (1); Brie (4); Limburger and other surface-ripened cheeses (6)
<i>Propionibacterium fruedenreichii</i> subsp. <i>shermanii</i>	Emmenthal and other Swiss type cheeses (4)
<i>Brevibacterium linens</i>	Camembert (3); Limburger and other surface-ripened cheeses (6); Smear ripened cheeses (9); Gubbeen (11)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

<i>Taxon</i>	<i>Products</i>
<i>Streptococcus thermophilus</i>	Cheddar (9)
<i>Lactobacillus helveticus</i>	Cheddar (9)
<i>Lactobacillus subsp. casei</i>	Cheddar (9)
<i>Lactobacillus casei</i> subsp. <i>pseudoplantarum</i>	Cheddar (9)
Non-starter Microorganisms (generally adventitious; poorly acidifying lactic acid bacteria, micrococci, corynebacteria, yeasts and molds)	
<i>Lactococcus raffinolactis</i>	Fior di Latte di Agerola (10)
<i>Lactococcus garvieae</i>	Artisanal Mozzarella (16); Fior di Latte di Agerola (10); Salers (25); Toma Piemontese (29)
<i>Streptococcus durans</i>	Kefir (1)
<i>Streptococcus filant</i>	Kefir (1)
<i>Streptococcus bovis</i>	Artisanal Mozzarella (16); Scamorza Altamurana(23); Ragusano (32)
<i>Streptococcus uberis</i>	Artisanal Mozzarella (16)
<i>Streptococcus parauberis</i>	Fior di Latte di Agerola (10)
<i>Streptococcus suis</i>	Fior di Latte di Agerola (10); Toma Piemontese (29)
<i>Streptococcus millieri</i>	Salers (25)
<i>Aerococcus viridans</i>	Artisanal Mozzarella (16)
<i>Enterococcus suphuranus</i>	Artisanal Mozzarella (16); Ragusano (32)
<i>Enterococcus hirae</i>	Ragusano (32)
<i>Enterococcus faecalis</i>	Stilton (31)
<i>Pediococcus acidilactici</i>	Parmigiano Reggiano (13); Ragusano (32)
<i>Pediococcus pentosaceus</i>	Cheddar (9); Salers (25)
<i>Lactobacillus casei</i>	Cheddar (6); Jarlberg (9); Norvegia (9); Hergard (9); Greve (9); Gouda (9); Irish Cheddar (9); Parmigiano Reggiano (13)
<i>Lactobacillus casei</i> subsp. <i>casei</i>	Italian ewe's cheeses (33)
<i>Lactobacillus casei</i> subsp. <i>pseudoplantarum</i>	Italian ewe's cheeses (33)
<i>Lactobacillus paracasei</i>	Jarlberg (9); Norvegia (9); Hergard (9); Greve (9); Gouda (9); Kefalotyri (9); Tenerife goat's milk cheese (9); Majorero (9); Arzua (9); Armada (9); Serra da Estrela (9); Casu Axedu (9); Fontina (9); Swiss type (9); British Cheddar (9); Irish Cheddar (9); Pecorino (15); Comté (24)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	Parmigiano Reggiano (13); Italian ewe's cheeses (33)
<i>Lactobacillus paracasei</i> subsp. <i>tolerans</i>	Parmigiano Reggiano (13)
<i>Lactobacillus rhamnosus</i>	Jarlberg (9); Norvegia (9); Hergard (9); Gouda (9); Cheddar (9); Grana Padano (12); Parmigiano Reggiano (13); Caciocavallo Silano (18); Comtè (24); Fiore Sardo (30); Italian ewe's cheeses (33)
<i>Lactobacillus plantarum</i>	Cheddar (6); Feta (9); Teleme (9); Kefalotyri (9); Tenerife goat's milk cheese (9); Cabrales (9); Afuega'l Pitu (9); Majorero (9); Mahon (9); Arzua (9); Armada (9); Serra da Estrela (9); Casu Axedu (9); Fontina (9); Toma (9); Swiss type (9); Irish Cheddar (9); Pecorino (15); Stilton (31); Italian ewe's cheeses (33)
<i>Lactobacillus paraplantarum</i>	Fiore Sardo (30)
<i>Lactobacillus brevis</i>	Afuega'l Pitu (9); Majorero (9); Swiss type (9); British Cheddar (9); Irish Cheddar (9); Caciocavallo Silano (18); Canestrato Pugliese (22); Italian ewe's cheeses (33)
<i>Lactobacillus fermentum</i>	Majorero (9); Toma (9); British Cheddar (9); Italian ewe's cheeses (33)
<i>Lactobacillus curvatus</i>	British Cheddar (9); Irish Cheddar (9); Fior di Latte di Agerola (10); Caciocavallo Silano (18); Fiore Sardo (30); Italian ewe's cheeses (33)
<i>Lactobacillus helveticus</i>	British Cheddar (9)
<i>Lactobacillus bifementans</i>	British Cheddar (9)
<i>Lactobacillus buchneri</i>	British Cheddar (9)
<i>Lactobacillus parabuchneri</i>	British Cheddar (9); Comtè (24)
<i>Lactobacillus farcininis</i>	British Cheddar (9)
<i>Lactobacillus kefir</i>	British Cheddar (9)
<i>Lactobacillus graminis</i>	Fior di Latte di Agerola (10); Fiore Sardo (30)
<i>Lactobacillus sakei</i>	Fior di Latte di Agerola (10); Caciocavallo Silano (18); Fiore Sardo (30)
<i>Lactobacillus homoiochii</i>	Fior di Latte di Agerola (10)
<i>Lactobacillus maltaromicus</i>	Fior di Latte di Agerola (10)
<i>Lactobacillus pentosus</i>	Fior di Latte di Agerola (10); Canestrato Pugliese (22); Fiore Sardo (30); Italian ewe's cheeses (33)
<i>Lactobacillus gasseri</i>	Scamorza Altamura (23)
<i>Carnobacterium divergens</i>	Artisanal Mozzarella (16)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
<i>Carnobacterium piscicola</i>	Artisanal Mozzarella (16)
<i>Brevibacterium imperiale</i>	Smear ripened cheeses (9)
<i>Brevibacterium fuscum</i>	Smear ripened cheeses (9)
<i>Brevibacterium oxydans</i>	Smear ripened cheeses (9)
<i>Brevibacterium helvolum</i>	Smear ripened cheeses (9)
<i>Corynebacterium casei</i>	Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11)
<i>Corynebacterium ammoniagenes</i>	Smear ripened cheeses *** (9)
<i>Corynebacterium betae</i>	Smear ripened cheeses *** (9)
<i>Corynebacterium insidiosum</i>	Smear ripened cheeses *** (9)
<i>Corynebacterium variabile</i>	Smear ripened cheeses *** (9); Milleens (11); Gubbeen (11)
<i>Arthrobacter arilaitensis</i>	Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11)
<i>Arthrobacter mysoarens</i>	Durrus (11)
<i>Arthrobacter citreus</i>	Smear ripened cheeses *** (9)
<i>Arthrobacter globiformis</i>	Smear ripened cheeses *** (9)
<i>Arthrobacter nicoitanae</i>	Smear ripened cheeses *** (9)
<i>Microbacterium gubbeenense</i>	Gubbeen (11)
<i>Agrococcus</i> sp. nov	Gubbeen (11)
<i>Curtobacterium poinsettiae</i>	Smear ripened cheeses *** (9)
<i>Microbacterium imperiale</i>	Smear ripened cheeses *** (9)
<i>Rhodococcus fascians</i>	Smear ripened cheeses *** (9)
<i>Caseobacter variabilis</i>	Smear ripened cheeses *** (9)
<i>Macrococcus caseoliticus</i>	Ragusano (32)
<i>Micrococcus</i> spp.	Limburger and other surface-ripened cheeses *** (6)
<i>Micrococcus luteus</i>	Smear ripened cheeses *** (9); Adrahan (11)
<i>Micrococcus lylae</i>	Smear ripened cheeses *** (9)
<i>Kocuria kristinae</i>	Smear ripened cheeses *** (9)
<i>Kocuria roseus</i>	Smear ripened cheeses *** (9)
<i>Staphylococcus epidermidis</i>	Durrus (11); Artisanal Mozzarella (16)
<i>Staphylococcus equorum</i>	Smear ripened cheeses *** (9); Milleens (11); Adrahan (11); Stilton (31)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
<i>Staphylococcus vitulus</i>	Smear ripened cheeses *** (9)
<i>Staphylococcus xylosus</i>	Smear ripened cheeses *** (9)
<i>Staphylococcus saprophyticus</i>	Smear ripened cheeses *** (9); Gubbeen (11)
<i>Staphylococcus carnosus</i>	Artisanal Mozzarella (16)
<i>Staphylococcus lentus</i>	Smear ripened cheeses *** (9)
<i>Staphylococcus sciuri</i>	Smear ripened cheeses *** (9)
<i>Halomonas venusta</i>	Milleens (11); Gubbeen (11); Adrahan (11)
<i>Saccharomyces cerevisiae</i>	Salers (26)
<i>Saccharomyces unisporus</i>	Salers (26)
<i>Candida utilis</i>	Smear ripened cheeses *** (9)
<i>Debaryomyces hansenii</i>	Roquefort (5); Camembert (7); Limburger and other surface-ripened cheeses *** (6); Smear ripened cheeses *** (9); Danish blue (9); Milleens (11); Gubbeen (11); Durrus (11); Adrahan (11); Water buffalo Mozzarella (8); Salers (26); Sardinian ewe's cheeses (28)
<i>Kluyveromyces lactis</i>	Roquefort (5); Camembert (7); Water buffalo Mozzarella (8); Smear ripened cheeses *** (9); Salers (26); Sardinian ewe's cheeses (28)
<i>Kluyveromyces marxianus</i>	Salers (26); Sardinian ewe's cheeses (28)
<i>Geotrichum candidum</i>	Smear ripened cheeses *** (9); Sardinian ewe's cheeses (28)
<i>Yarrowia lipolytica</i>	Camembert (7) Limburger and other surface-ripened cheeses *** (6); Danish blue (9); Milleens (11); Sardinian ewe's cheeses (28)
<i>Brettanomyces</i> spp.	Water buffalo Mozzarella (8)
<i>Dekkera anomala</i>	Sardinian ewe's cheeses (28)
<i>Candida</i> spp.	Roquefort (5); Camembert (7); Limburger and other surface-ripened cheeses *** (6)
<i>Candida zeylanoides</i>	Salers (26)
<i>Candida parapsilosis</i>	Salers (26)
<i>Candida silvae</i>	Salers (26)
<i>Candida intermedia</i>	Salers (26)
<i>Candida kefir</i>	Kefir (1)
<i>Candida catenulata</i>	Sardinian ewe's cheeses (28)

(continued)

Table 2.1 Microorganisms in Main Fermented Dairy Products on the Basis of Selected Literature Referred to Representative Products of Different Type (continued)

Taxon	Products
<i>Candida rugosa</i>	Danish blue (9); Salers (26)
<i>Candida sake</i>	Sardinian ewe's cheeses (28)
<i>Candida glabrata</i>	Danish blue (9)
<i>Zygosaccharomyces</i> spp.	Danish blue (9)
<i>Pichia guilliermondii</i>	Salers (26)
<i>Pichia fermentans</i>	Sardinian ewe's cheeses (28)
<i>Pichia membranefaciens</i>	Sardinian ewe's cheeses (28)
<i>Rhodotorula rubra</i>	Sardinian ewe's cheeses (28)
<i>Penicillium</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9); Taleggio *** (9)
<i>Mucor</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9); Taleggio *** (9)
<i>Cladosporium</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9)
<i>Geotrichum</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9)
<i>Cryptococcus laurentii</i>	Sardinian ewe's cheeses (28)
<i>Issatchenkia orientalis</i>	Sardinian ewe's cheeses (28)
<i>Epicoccum</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9)
<i>Sporotrichum</i> spp.	St. Nectair *** (9); Tome de Savoie *** (9)

*For cheeses produced without starter addition or back-slopping practice, we intended as "starter" those microorganisms well-known as acidifying or fla-

voring agents

*** "Ropy" or "slime" variants

*** The surface microflora is fundamental for the typical quality of this cheese variety. In some cases it is exclusively adventitious; in some other "smears" of old cheeses are utilized as adjunct for promoting ripening of young cheeses. Therefore in these last cases the microbial species could be included among co-starters or adjuncts

- (1) Oberman and Libudzisz, 1998; (2) Tamime and Robinson, 1999; (3) Johnson and Steele, 1997; (4) Stanley, 1998; (5) Besancon, et al. 1992; (6) Johnson 1998; (7) Nooitgedagt and Hartog 1988; (8) Coppola, et al. 1988; (9) Beresford, et al. 2001; (10) Coppola, et al. 2006; (11) Mounier, et al. 2005; (12) Lazzi, et al. 2004; (13) Coppola R., et al. 2000; (14) Bottazzi 1993; (15) Coda, et al. 2006; (16) Morea, et al. 1999; (17) Morea, et al. 1998; (18) Pirano, et al. 2005; (19) Tsakalidou, et al. 1998; (20) Pacini, et al. 2006; (21) Mucchetti and Neviani 2006; (22) Aquilanti, et al. 2006; (23) Baruzzi, et al. 2002; (24) Berthier, et al. 2001; (25) Callon, et al. 2004; (26) Callon, et al. 2006; (27) Cosentino, et al. 2004; (28) Cosentino, et al. 2001; (29) Fortina, et al. 2003; (30) Mannu, et al. 2000; (31) Ercolini, et al. 2003a; (32) Randazzo, et al. 2002; (33) De Angelis, et al. 2001

viable and active microorganisms. Extensive genomic sequencing of dairy microorganisms will be able to detect increasing numbers of new targets to monitor, and will allow considerable progress in describing microbial genetic diversity and its potential functional activity in fermented dairy products. It must, however, be recognized that only combined efforts of these approaches with proteomics, chemiometric measurements and sensorial evaluation can elucidate to an exploitable extent the complex and dynamic processes briefly discussed herein.

2 The Use of Molecular Methods in Dairy Microbiology

Approaches to studying microorganisms in food have undoubtedly changed. Advances in molecular biology have provided more information on food-associated bacteria and have also provided the scientific community with sound, reliable and effective methods for detection, identification and typing of microorganisms from food. The availability of such methods has made food scientists shift from a more traditional isolation and biochemical characterization of microbes from food, to a direct detection of microbes – not as microbes, but rather as “D/RNA from microbes” themselves. How have dairy microbiologists made use of these novel approaches so far? The main interest of dairy microbiologists is to study the diversity and dynamics of microorganisms in dairy produce and possibly to correlate the occurrence of certain microbial species and strains with desired flavor and sensorial traits of the products.

Various molecular methods can be used depending on the level of information required by research. Dairy microbiologists can be interested in identification, detection or typing. Identification and detection can benefit from the availability of both culture-dependent and culture-independent techniques, whereas typing is an analysis performed on isolates and is, thus, strictly related to culture-dependent methods.

Identification can be carried out at different levels. The dairy microbiologist can be interested in classifying his microbiota of interest at genus, species and sometimes strain level. Of course the methods to be employed can vary each time.

2.1 Culture-independent Approaches

Identification at genus/species level can be achieved by using culture-independent techniques such as PCR-DGGE/TGGE/SSCP. These methods have the advantage of providing identification and monitoring of a microbiota at species level without isolating the microorganisms on culture media. Instead of isolating bacteria of each sample from milk, natural starter, intermediate of production or the final cheese, direct DNA extraction can be achieved to provide a mixture of nucleic acids from most of the microorganisms present in the original dairy matrix. PCR amplification

is subsequently required, and the most commonly employed target for identification at species level is the DNA encoding for ribosomal RNA. In most cases, for the identification of *Bacteria*, portions of the 16S rRNA gene are used. The 16S rRNA gene is conserved and allows the development of PCR primers that can be used for all *Bacteria*. However, it also contains variable regions, whose variability of sequence is species-specific in most cases. Therefore, the result of PCR amplification will be a portion of the 16S rRNA gene from all the microbial species whose DNA was extracted *in situ*, and the sequence of the amplicon is likely to vary from species to species. This sequence variation will allow separation of the fragments according to formation of discrete regions of thermal (TGGE) or chemical (DGGE) denaturation or the formation of different single strand conformations (SCCP), but the final output of the analysis will always be a fingerprint. The fingerprint will be made of a number of bands corresponding (in most but not all cases) to as many microbial species and will represent the microbiological identity of the milk, starter, intermediate of production or cheese analyzed. The final identification of each species can then be obtained by the purification and sequencing of each band and by comparison with the available data bases (Gene Bank, www.ncbi.nlm.nih.gov/blast/).

2.1.1 Diversity and Dynamics of Natural and Selected Starter Cultures

The microbes occurring in the cheese may arise from the raw milk, from the environment and tools of production, or can be added as selected starter cultures under controlled conditions. Another interesting possible source of bacteria exists in natural starter cultures, currently employed in much traditional cheese making where fermentation is assured by the back-slopping of milk or whey cultures from previous preparations.

The studies of selected and natural starter cultures share common interests such as the knowledge of the fate of microorganisms present in the culture at the beginning of fermentation, and their interaction with background microbiota. These interactions are recognized to be fundamental in selecting the microorganisms actually dominating the process and contributing to the principal rheological and sensorial attributes of the cheese. In addition, the study of the accessory microbiota is also important since the bacteria occurring at lower loads, along with the dominant bacteria, can potentially contribute to the development of product flavor and taste, thanks to specific metabolic pathways (Beresford, et al. 2001).

Of the above-cited fingerprinting techniques the most commonly employed in dairy microbiology is PCR-DGGE. We will be giving some examples of how this has been used to study the diversity and dynamics of microbial communities in cheese production.

The PCR-DGGE approach has been exploited to directly identify microbial species occurring in natural whey cultures (NWCs) used as starter for water buffalo Mozzarella cheese manufacture (Ercolini, et al. 2001). Both thermophilic and mesophilic LAB were identified by sequencing of the V3 region of the 16S rRNA

gene from DGGE fragments of NWCs profiles, namely *Lb. delbrueckii*, *Lb. crispatus*, *Lactococcus (L.) lactis* and *Streptococcus (St.) thermophilus*. Moreover, the occurrence of contaminants such as *Alishewanella fetalis* could also be highlighted. In the same study, a novel PCR-DGGE approach was developed to rapidly check the diversity of the bacterial community after cultivation on specific or non-specific culture media. Briefly, after colony counting has been performed, the colonies from the plates can be collected in “bulks” and subjected to DNA extraction and PCR-DGGE analysis (Ercolini, et al. 2001). Consequently, a DGGE fingerprint can be obtained for each plate, dilution, and culture medium. This method to investigate the cultivable microbial community has been shown to have good potential in food microbiology (Ercolini, et al. 2001; Ercolini, et al. 2003a; Ercolini, et al. 2004; Ercolini 2004). Firstly, it provides an alternative to traditional tools for identification. Qualification of the dominant species could be achieved by sequencing of the DGGE bands arising from the patterns corresponding to the highest dilutions, in spite of the isolation of single colonies followed by purification and biochemical identification (Ercolini 2004). Analysis of DGGE profiles obtained from bulk cells provides an image of the cultivable community, while simultaneously allowing ecological information to be obtained. Ercolini, et al. (2001) counted a population of mesophilic streptococci of 10^8 cfu/ml in NWCs for Mozzarella cheese production, but realized, after bulk PCR-DGGE analysis of all the dilutions, that the only species reaching the value of 10^8 was the thermophilic *St. thermophilus* and that mesophilic cocci were only present at levels of 10^4 cfu ml⁻¹.

PCR-DGGE fingerprinting can also be useful to trace process dynamics during cheese making. The approach can be used to track the starter during production by examining the fingerprints of samples from raw material, through intermediate of production until the final product. This is important in both traditional and industrial dairy production. In the latter case the use of selected starter cultures ensures controlled fermentation and a standard quality of the final product. Analysis of DGGE fingerprints of the samples during manufacture can be important to ascertain that the starter culture is actually dominating the fermentation and can be of help in highlighting the occurrence of contaminating bacteria. On the other hand, in the case of traditional cheese production, one can trace the evolution of the contributing microbiota during the whole production and assess whether the raw milk or the natural whey/milk culture microflora actually contributes to cheese production. It can also show which microbial species of the natural starter survives fermentation, processing and the possible stresses imposed by the technology of production (pH, thermal stress, etc.). In a recent study the fate of the natural whey culture for the manufacture of traditional water buffalo Mozzarella cheese was investigated by PCR-DGGE (Ercolini, et al. 2004). The analysis of DGGE fingerprints from the intermediate samples during cheese production was shown to be useful to check the natural starter effectiveness and to determine the contribution of different groups of LAB during fermentation leading to the final Mozzarella cheese. All the DGGE profiles of dairy samples taken during manufacture were analyzed: raw milk, NWC, raw milk after NWC addition, curd before and after ripening, drained whey, stretched curd and final product (Ercolini, et al. 2004). A single

glance at the succession of the fingerprints (Fig. 2.1) explains all that occurred in the process: the raw milk had a complex profile, but none of the species occurring in the milk were present in the profiles of the other samples. As soon as the NWC was added to the milk, the profile changed into the NWC fingerprint, which was displayed by all further samples until the final water buffalo Mozzarella cheese (Fig. 2.1). In other words, in this specific manufacture, the NWC was the main performer in the fermentation, giving high loads of bacteria to the raw milk, concealing the raw milk microbiota in the fingerprints, but probably giving strength to the fermentation and allowing the process to be properly carried out in respect of tradition. In this case, the microbial succession could be registered as “pictures” of microbial groups involved in premium quality production. This procedure may find useful applications for the monitoring of non-premium quality products where poor quality arises from the lack of development of the NWC. This procedure can be easily applied to dairy plants, allowing process development and starter effectiveness to be checked by analyzing dairy samples by PCR-DGGE. In comparison with traditional culture-dependent microbiological analyses, molecular approaches can be considered a step forward for the innovation of tracing systems in food technology, and may play an important role in the quality control of traditional

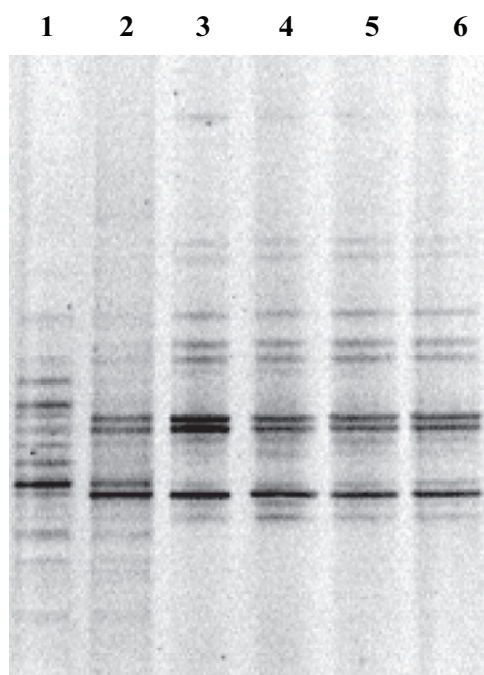


Fig. 2.1 PCR-DGGE profiles of dairy samples during water buffalo Mozzarella cheese manufacture. Lanes: 1, raw milk; 2, milk after NWC addition; 3, NWC; 4, curd after ripening; 5, stretched curd; 6, final product. (From Ercolini, et al. (2004), permission granted)

products, allowing preservation of their typical identity and consumer protection when territory claims are involved.

The dynamics of complex starter mixture can also be monitored to assess whether they are influenced by certain technological interventions in a cheese making process. Recently, PCR-DGGE was also applied to check the response of the smear microbiota of smear-ripened cheese to the use of a bacteriocin-producing culture used as protective adjunct to combat the occurrence of *Listeria monocytogenes* (O'Sullivan et al. 2006). The authors proved that there was apparently no effect on the smear microbial flora of the cheeses treated with the bacteriocin-producing culture in comparison with the untreated control (O'Sullivan et al. 2006). Even though PCR-DGGE profiling is not always very precise in describing all the species occurring in a certain environment (Ercolini, 2004), in cases such as this it can be useful to assess whether or not the microbiological state of a starter has changed, according to environmental and technological variations. Again, the approach to studying dairy cultures has changed since, in such cases, traditional analysis of the microbiota would have been performed by plate counts, isolation followed by biochemical identification, etc., to have information on the response of a certain flora to varied conditions.

Fingerprinting techniques can also be used to characterize wide numbers of natural starter cultures, especially when they are used by different dairies of a particular geographic region to produce the same type of cheese. Effective application of PCR-DGGE for grouping NWCs was reported by Mauriello, et al. (2003). In this study PCR-DGGE analysis was used for discriminating natural starters for traditional water buffalo Mozzarella cheese production that were sampled from different dairies in southern Italy. The profiles showed that the microbial composition of the starters was strongly dependent on their geographical origin, with starters from the same area displaying closely related DGGE profiles. It was also demonstrated that the flavors (detected by chromatographic methods) potentially provided by the development of each starter during curd ripening were linked to the complexity of the microbial flora shown by DGGE, and thus to the geographical origin of the products (Mauriello, et al. 2003). This constitutes evidence that the microbial diversity of natural starter cultures and its evolution during fermentation may represent important proof of authenticity for the traceability of origin and mode of production of traditional dairy products.

2.1.2 Diversity and Dynamics of Microbial Populations in Cheese and during Cheese Manufacture

The same approach used for monitoring the fate of starter cultures during cheese production can be used to obtain structure and identification of microbial communities in cheese and during cheese manufacture.

Randazzo, et al. (2002) examined the microbial succession in manufacturing of Ragusano, an artisanal Sicilian cheese. The variable region V6-V8 of the 16S rRNA gene was used in DGGE analysis to identify the total microflora, while specific

primers for *Lactobacillus* were employed spanning the V1-V3 region. Analysis of the active microflora was also performed by 16S rRNA RT-PCR followed by DGGE. DGGE profiles from samples taken during cheese production indicated dramatic shifts in the microbial community structure. Cloning and sequencing of rRNA gene amplicons revealed that mesophilic bacteria, including leuconostocs, *L. lactis* and *Macrococcus caseolyticus*, were dominant in the raw milk while *St. thermophilus* prevailed during fermentation. Other rod-shaped LAB, especially *Lactobacillus fermentum* and *Lb. delbrueckii*, were also found during ripening. Moreover, the authors found that *Lb. delbrueckii* was not cultivable, while some isolated species of enterococci and pediococci could be not found in the DGGE profiles.

The bacterial community occurring in Stilton cheese was structured by PCR-DGGE and sequencing of the 16S rRNA regions V3 and V4-V5 (Ercolini, et al. 2003a). The traditional British PDO cheese was shown to be colonized by a complex microbial flora including *Lb. plantarum*, *Lb. curvatus*, *L. lactis*, *Staphylococcus* (*S.*) *equorum*, *Enterococcus* (*E.*) *faecalis*, *Leuconostoc* (*Lc.*) *mesenteroides*. It was found that microbial diversity revealed from the same DNA templates amplifying two different regions of the 16S rRNA gene could be different. Indeed, the presence of *Leuconostoc* in Stilton cheese was revealed only by analyzing the V4-V5 region of the 16S rRNA gene while the species was not detected when the V3 region was targeted (Ercolini, et al. 2003a). Targeting more than one variable region may potentially widen the microbial diversity detected but could be more time-consuming. However, even if one region is targeted, other experiments should be done to ascertain whether other microbial species are present, but not detected.

The comparison of PCR-DGGE profiles of different cheeses of the same category, or of the same cheese manufactured by different procedures, can help assess the behavior of starter bacteria or adventitious microbial flora. For example, Randazzo, et al. (2006) found that the dominant bacteria in the manufacture of Pecorino Siciliano cheese were *St. bovis* and *L. lactis*, although various cheese making procedures were tested. In addition, Obodai and Dodd (2006) showed that *Lb. delbrueckii* subsp. *delbrueckii* and *St. thermophilus* were the principal bacterial species involved in the production of nyarmie, a Ghanaian fermented milk product obtained by natural fermentation. The authors concluded that these thermophilic bacteria, or alternatively mesophilic bacteria, could be selected as starter cultures in order to improve and/or standardize the quality of Nyarmie (Obodai and Dodd 2006).

Another culture-independent fingerprinting technique, very similar in principle to PCR-DGGE, is PCR-TTGE. This method has been used on several occasions to describe dairy ecosystems (Ogier, et al. 2002; Lafarge, et al. 2004; Ogier, et al. 2004; Parayre, et al. 2007). Initially, the technique was used to set up a species database in which each species or group of species was characterized by a specific TTGE fingerprint (Ogier, et al. 2002). The variable V3 region of the 16S rRNA gene of about 50 microbial species possibly occurring in dairy ecosystems and belonging to the genera *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*,

Streptococcus, *Weissella* and *Staphylococcus* was analyzed in TTGE to develop the database. However, TTGE fingerprints characterized by multiple bands for one species were highlighted. In addition, cases of TTGE co-migration of V3 amplicons from different species were also found within the *Lb. acidophilus* and *Lb. plantarum* groups, and within the species of *Leuconostoc* and *Enterococcus* (Ogier, et al. 2002). In fact, the latter evidence can represent a problem in obtaining reliable identification of microbial species by simple comparison of TTGE bands in a cheese fingerprint with the migration distances of the species of the database.

Use of the migration map to identify dairy bacteria was validated by analyzing dairy preparations from defined microbial content to increasing microbial complexity; in the latter samples bands that could not be recognized in the migration field of the species of the database were identified by cloning and sequencing, and were often shown to be Gram negative contaminants (Ogier, et al. 2002). In a further study, Ogier and co-workers (2004) extended the database to 150 microbial species, including possible contaminants and spoilage bacteria. The database of high G+C% bacteria was set up by DGGE analysis of V3 amplicons. The authors analyzed several dairy products such as Morbier, Munster, Epoisses and Leerdamer Swiss cheese, identifying a large number of bacteria by using their database and, in cases of unidentified bands, by cloning and sequencing the fragments and/or using species-specific PCR assays to sort out uncertainty in some identifications (Ogier, et al. 2004). As expected, it was found that raw milk cheeses such as Morbier were richer in microbial diversity than Leerdamer cheese obtained from pasteurized milk. It was shown that while LAB dominated in the core of the cheese, high G+C% coryneform bacteria such as *Corynebacterium variabile*, *C. mastitidis*, *C. casei*, *Arthrobacter* spp. and *Brevibacterium linens* could be identified from the surface of the cheeses (Ogier, et al. 2004). This is further confirmation that micro-environments characterized by different ecological factors can develop across a cheese matrix and, therefore, a heterogeneous spatial distribution of microbial species can occur at the end of ripening, as also shown by 16S rRNA FISH analysis of Stilton cheese by Ercolini, et al. (2003a). Recently, a PCR-TTGE approach was used for the optimization of a DNA extraction method from dairy products with a fingerprint reproducibility of 89 percent (Parayre, et al. 2007).

An original approach has also been developed to achieve a culture-independent microbial characterization of dairy samples, based on direct DNA/RNA extraction followed by PCR amplification and SSCP analysis by capillary electrophoresis. This approach was used on several occasions to study the microbial diversity of Salers, a Registered Designation Origin (RDO) semi-hard cheese from raw milk produced in France (Duthoit, et al. 2003, 2005a and 2005b; Callon, et al. 2006). Duthoit, et al. (2003) established their most suitable conditions for DNA extraction and employed different targets for 16S rRNA gene amplification to highlight the presence of LAB and high G+C% bacteria. An initial cloning strategy of the PCR products from curd, followed by sequencing and screening of the clone library by SSCP, was adopted to identify the different

SSCP peaks and to be able to recognize the corresponding microbial species in the analysis of cheese during ripening. LAB such as *L. lactis*, *St. thermophilus*, *E. faecium*, *Lc. paramesenteroides*, *Lc. mesenteroides*, *Lb. plantarum* and *Lb. pentosus* were identified; in addition, high G+C% corynebacteria such as *C. bovis*, *C. variabilis*, *C. afermentans* and *C. flavescentis* were detected (Duthoit, et al. 2003). The authors showed that different results may be obtained by amplifying the V2 or V3 regions of the 16S rRNA gene, and that different microbial species may give the same migration properties in PCR-SSCP. Moreover, the identified species showed different trends according to cheese production (from different dairies) and time of ripening. *Enterobacteriaceae* occurred in raw milk, but disappeared during curd ripening, while the proportion of LAB species varied according to sample and time of ripening.

The diversity of the microbial populations in Salers cheese was also assessed by using RNA as a template in RT-PCR to obtain fingerprints of the active microbiota (Duthoit, et al. 2005a and 2005b). By comparing DNA and RNA SSCP profiles the authors realized that the revealed microbial species were different and that the active microbial species found in SSCP profiles did not always match those detected by DNA-based PCR-SSCP. The authors concluded that RNA-based SSCP was more pertinent than DNA-based SSCP to measure the diversity of the microbial community of Salers (Duthoit, et al. 2005b). The comparisons of microbial diversity were based on the calculation of diversity indexes, often taking into account the peak ratios of the different microbial species (Duthoit, et al. 2003, 2005a and 2005b). However, this strategy may be significantly biased by selective PCR amplification phenomena as often reported (Reysenbach, et al. 1992; Suzuki and Giovannoni 1996; Ercolini, et al. 2001b; Ercolini 2004), and also by the abundance of the number of rRNA gene copies (Farrelly, et al. 1995). Therefore, the dominance of a particular peak/species may be due to its selective amplification in PCR and not to its actual abundance in that particular dairy sample (Ercolini 2004).

The microbial diversity detected in Salers cheese by PCR-SSCP was tentatively compared to the sensorial properties of the cheese (Duthoit, et al. 2005a). The sensorial attributes of the cheese were shown not to be correlated to the producer, but to be influenced by ripening time. Changes in sensorial properties during ripening could be correlated to the variation in the RNA-based SSCP profiles. Moreover, bacteria not usually considered of technological interest – such as corynebacteria, *Enterobacteriaceae*, *Bacillus* spp., and some unidentified SSCP peaks – were shown to probably be involved in the development of texture, taste, flavor or aroma (Duthoit, et al. 2005a). However, the general profiling of the bacterial population at species level by SSCP fingerprinting cannot provide enough information on the sensorial quality of Salers cheese, which remains, as for most cheeses, fairly unpredictable. Diversity at strain level in metabolic activities (Giraffa, et al. 2001) and aroma production (Mauriello, et al. 2001) must be investigated to enhance our knowledge of how microbial succession affects the sensorial quality of cheeses.

The PCR-SSCP was also developed to identify staphylococcal populations in dairy products (Delbes and Montel 2005). A nested-PCR assay was developed by

using primers for selective amplification of the 16S rRNA gene of staphylococci, and SSCP analysis was applied to identify the staphylococci during the production of raw milk cheeses. *S. equorum*, *S. saprophyticus* and *S. aureus* were detected as dominant species in the dairy samples (Delbes and Montel 2005). Moreover, the amplicons from *S. aureus* were always found to be more abundant than the other species, even when equal amounts of DNA templates were used, probably due to preferential PCR amplification or 16S rRNA copy number heterogeneity (Coenye and Vandamme 2003).

Different yeast species can also occur in dairy products and can play a role in curd ripening and aroma development. The PCR-SSCP approach was also implemented to profile the yeast community in Salers cheese (Callon, et al. 2006). Yeast-specific primers were designed to amplify the V4 region of the 18S rRNA gene for SSCP analysis. The yeast species most frequently found in Salers were *Kluyveromyces lactis*, *K. marxianus*, *Candida zeylanoides*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae* (Callon, et al. 2006).

Overall, the SSCP approach may be useful for profiling the microbial populations in dairy products and to observe the marked variation in microbial species composition during ripening. However, identification of the microbial species based on the co-migration of the PCR amplicons of reference species may be unsatisfactory since the occurrence of certain bacteria in the dairy sample is not always predictable, and also due to possible co-migration of amplicons from different species.

2.1.3 Microbial Profiles of Dairy Products for Quality Assessment

PCR-DGGE applied to template DNA directly extracted from a food matrix generates a specific profile of that product in that moment, given the conditions used. The fingerprint gives a “picture” of the microbiota of the product and can be taken into account as a specific trait of that food just like other biochemical, structural or sensorial properties. PCR-DGGE fingerprinting of food and drinks has been tested by several authors who discovered it can identify the microbiological traits of food products and may represent a tool for quality control.

Coppola, et al. (2001) discriminated between industrial and artisanal pasta filata cheeses by comparing the DGGE profiles of different commercial products. The authors found that PCR-DGGE was better than 16S-23S intergenic spacer region analysis at differentiating pasta filata cheeses. Cluster analysis of the fingerprints showed the dairy products grouped according to their microbial complexity; it was found that traditional pasta filata cheeses had profiles which were rich in bands, and that the degree of complexity of the microbial flora decreased when the product was of industrial manufacture. On the basis of their results, Coppola, et al. (2001) suggested that PCR-DGGE can be considered valid for discriminating traditional and industrial cheeses – also for legal purposes – when products obtained through prescribed manufacturing regulations are analyzed. The potential of PCR-DGGE in differentiating dairy products was further confirmed by Ercolini, et al. (2002) through profiling different kinds of

cheese, and showing them to be very different from each other. Unfortunately, it was also demonstrated that different samples of the same category of cheese could display different DGGE profiles (Ercolini, et al. 2002), thus compromising the use of the technique to develop class-specific profiles for cheese classification.

Dairy products with a defined microbial flora, produced by using starter cultures and/or in controlled conditions, are the easiest matrices to control as they usually display a simple DGGE profile where each band corresponds to the species expected to be there. Among these are yogurts and probiotic beverages or preparations. Recently, PCR-DGGE was shown to be effective in corroborating the occurrence of certain microbial species in yogurt and lyophilized probiotic preparations (Fasoli, et al. 2003; Temmerman, et al. 2003). A general congruence between microorganisms declared on the label and those revealed by PCR-DGGE was found by Fasoli, et al. (2003) for probiotic yogurts. However, the authors also found some discrepancies for probiotic preparations such as incorrect identification of some *Bacillus* and *Bifidobacterium* species and the presence of microbial entities not declared in the label (Fasoli, et al. 2003). These results are consistent with those obtained by Temmerman, et al. (2003) in analyzing several probiotic preparations by PCR-DGGE; the authors found the technique very useful to ascertain the occurrence of probiotics in the analyzed matrices, especially if compared to lengthy and uncertain traditional procedures. The PCR-DGGE control of such products rapidly and reliably revealed a rather high percentage of incorrectly labeled probiotic products, and viable counts often showed low loads of the declared species, thus compromising the probiotic value of the products (Temmerman, et al. 2003).

2.1.4 Specific Location of Microbial Colonies in Cheese by FISH

A further possible goal in microbial ecology can be to examine the spatial distribution of the bacteria within a cheese matrix. This can be achieved by means of fluorescence *in situ* hybridization (FISH) using 16S rRNA probes (Bottari, et al. 2006).

First of all, the FISH experiments apply to thin cheese sections that can endure the hybridization procedure. This can be achieved by using a polymerizing glycol methylacrylate resin to embed the cheese specimen to be cut in thin sections. This method was developed by Ercolini, et al. (2003b) and proved to be a successful alternative to cryo-sectioning for the achievement of down to 5 μ m dairy sections withstanding the hybridization buffers.

16S rRNA probes were developed for the specific detection of *L. lactis*, *Lc. mesenteroides* and *Lb. plantarum* to locate their colonies in Stilton cheese (Ercolini, et al. 2003a). A location and differential distribution of the microbial species was shown by FISH in the core, underneath the crust, and along the veins of Stilton cheese (Ercolini, et al. 2003a). The combined use of the universal Eub338 (Amann, et al. 1990) probe and the specific probes developed in FISH experiments

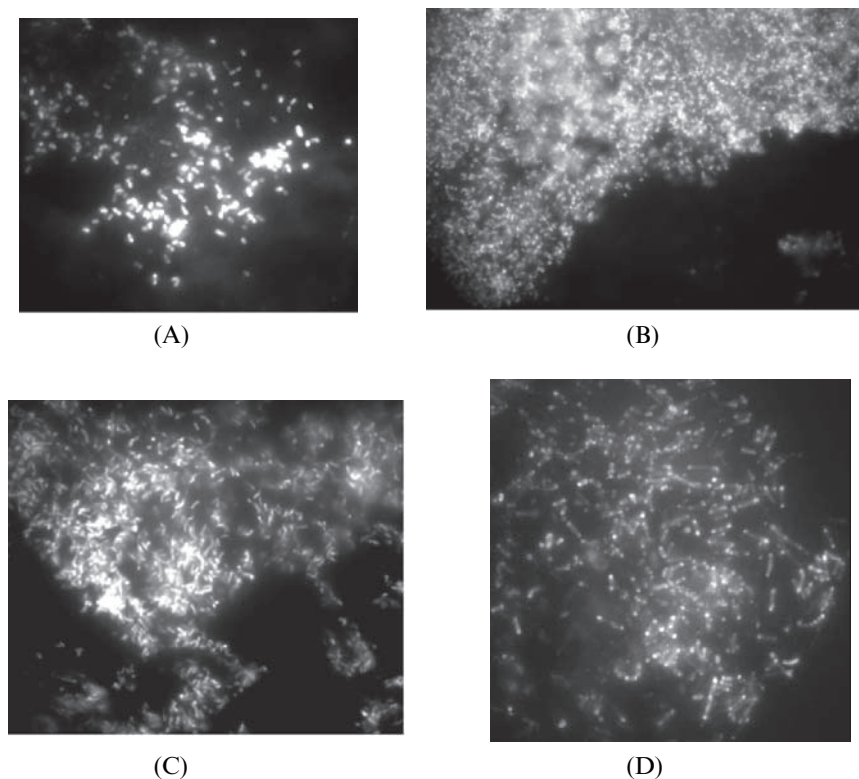


Fig. 2.2 Fluorescence *in situ* hybridisation of Stilton cheese sections (A) *L. lactis* microcolony from the core detected by using a *L. lactis* specific probe; (B) Microcolony of cocci along the vein detected by the universal probe Eub 338; (C) Colony of rods underneath the veins detected by probe Eub 338; (D) Microcolony of *Lb. plantarum* underneath the crust detected by using the *Lb. plantarum* specific probe. Adapted from Ercolini, et al. (2003a)

on Stilton cheese sections showed a differential spatial distribution of the bacterial flora within the dairy matrix (Fig. 2.2). A significant difference was detected between the core and the rest of the cheese; the former being much less rich in bacterial colonies. The colony density in the core was about 5-fold lower than the surface and the veins, although the micro-environment in the latter two was out-competed by mold development. In the core most of the microcolonies were *L. lactis* even though a considerable amount of *Lc. mesenteroides* were also detected. No rod-shaped bacteria were found in the core; instead, conspicuous amounts of *Lb. plantarum* were found underneath the crust (Ercolini, et al. 2003a). Across the veins, moreover, a few micro-colonies of *Lb. plantarum* could be observed while

micro-colonies of shorter rods resembling *Lb. curvatus* were much more abundant. On the surface, and much more along the internal margin of the veins, very large colonies of non-*Lactococcus* cocci were detected and supposed to be staphylococci even though identification has not been achieved.

The differential spatial distribution of microbial species in cheese has a major impact on knowledge in dairy science. The location of the different species in different zones of the matrix implies differential utilization of the dairy nutrients, consequently affecting the impact of flavor to the cheese which might result in a pool of various compounds that are not released homogeneously from the cheese, but arise from different sites with different microbial activity. Moreover, the production of anti-microbial compounds is also localized, thus affecting flora development. When poor quality products arise it may be due to the lack of development of one or more of these micro-environments.

3 Molecular Identification and Characterization of Microbial Strains Isolated from the Dairy Environment

Cultivable microflora of milk and dairy products is mainly represented by LAB (*Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Wiessella* and *Pediococcus*). However, strains of other genera such as *Propionibacterium*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium*, yeasts and molds can also occur. Today (November 2006; see also www.bacterio.cict.fr), the genus *Lactobacillus* contains 119 species, the genus *Streptococcus* 67 species, the genus *Enterococcus* 34 species, the genus *Lactococcus* 5 species, the genus *Leuconostoc* 14 species, the genus *Wiessella* 11 species, the genus *Pediococcus* 11 species and the genus *Propionibacterium* 13 species. The main microbial species and their occurrence in dairy products are highlighted in Table 2.1.

The problems of traditional identification methods, even when based on miniaturized easy-to-handle kits or devices, make their use difficult for a reliable identification or biochemical typing of microbial taxa from food.

For these reasons, significant efforts have been made to develop alternative identification methods combining speed, reliability and low cost. These criteria are met by methods based on molecular rather than phenotypic traits. The greatest advantage of DNA-based identification techniques is that these methods focus on the unique nucleic acid sequence of the microorganisms rather than on the phenotypic expression of products that are encoded by the respective genes. Polyphasic taxonomy, however, combines pheno- and genotypic information and forms the basis for current systematic bacteriology.

Moreover, new microbial species are continually being classified, making further identification tools necessary. Therefore, molecular methods have been increasingly used in order to simplify characterization procedures, to provide rapid and reliable identification, or to validate phenotypically determined taxa. Indeed, thanks to the results of the application of molecular tools, in the course

of 2006 new species of LAB were described in the genus *Lactobacillus* (Aslam, et al. 2006; Konstantinov, et al. 2006; Osawa, et al. 2006; Rodas, et al. 2006; Vancanneyt, et al. 2006a), *Enterococcus* (Carvalho, et al. 2006; Svec, et al. 2006), *Streptococcus* (Glazunova, et al. 2006), *Leuconostoc* (Chambel, et al. 2006) and *Pediococcus* (Franz, et al. 2006). By contrast, in the same period, some species of *Lactobacillus* (Naser, et al. 2006a-b; Dellaglio, et al. 2006; Felis, et al. 2006), *Enterococcus* (Naser, et al. 2006c) and *Leuconostoc* genera (Vancanneyt, et al. 2006b) were re-classified.

The primary objectives of food microbiological analysis are the control of food quality, food preservation, evaluation of starter culture efficiency, and monitoring of particular species/strains during manufacturing. With reference to the development of starter cultures, with consistent and predictable performance, it is widely recognized that extensive characterization of the strains and more detailed knowledge of their physiology, metabolism and genetics are required. Moreover, the increasing number of available commercial strains used as starters requires reliable methods to accurately differentiate strains at both species and biotype levels in pure and mixed cultures in order to defend rights and eliminate risks of confusion during their use.

The taxonomic level of microbial discrimination depends on the sensitivity of the technique used and may range from genus (or species) to strain level (sub-typing or strain typing). The ability of a molecular typing system to discriminate among genetically unrelated isolates is a reflection of the genetic variation seen in the chromosomal DNA of the bacterial species. Usually this variation is high, and differentiation of unrelated isolates can be accomplished using any of a variety of techniques. However, often technologically important traits of dairy microorganisms are not uniformly distributed within a species. Thus, the most important biotypes are often a smaller subset of the many strains that constitute a species. As a consequence, this subset may exhibit relatively little genetic diversity, and it can be difficult to differentiate among strains even with molecular techniques.

Type-ability refers to the ability of a technique to assign an unambiguous result (type) to each isolate. Non-type-able isolates are more common with phenotypic methods, but can also occur with genotypic methods. The reproducibility of a method means the ability to yield the same result upon repeat testing of a bacterial strain. Poor reproducibility may reflect technical variation in the method or biological variation occurring during in vivo or in vitro passage of the organisms to be examined. The discriminatory power of a technique refers to its ability to differentiate among unrelated isolates, ideally assigning each to a different type. In general, phenotypic methods have less discriminatory power than genotypic methods. Most molecular methods require costly material and equipment, but are relatively easy to learn and are applicable to variety of species. On the other hand, phenotypic methods also involve costs in labor and material and are restricted to a few species (sero-typing, phage-typing). Characteristics of some typing systems are reported in Table 2.2.

Although the classical phenotype-based (biotyping) methods are still of importance for daily routine analyses, genotypic methods have increasingly contributed to the in-depth characterization of microorganisms and their differentiation. It may be assumed that the combination of different fundamental and advanced methods

Table 2.2 Characteristics of Some Systems for Typing of Dairy Bacteria

Typing systems	Proportion of strains typeable	Reproducibility	Discrimination Power	Ease of Interpretation ¹	Ease of Performance	Cost (Time and money)
Phenotypic biotyping	All	Poor	Poor	Moderate	Easy	High
Antimicrobial susceptibility patterns	All	Good	Poor	Easy	Easy	Moderate
Serotyping	Low	Good	Fair	Moderate	Moderate	Moderate
Plasmid profiling	Most	Good	Good	Moderate	Good	Moderate
REA-CE	All	Good	Good	Difficult	Good	Low
REA-PFGE	All	Excellent	Excellent	Good	Moderate	High
RLFP (including ribotyping)	All	Excellent	Moderate to Excellent	Moderate	Difficult	High
RAPD-PCR (including its variants)	All	Good	Good	Moderate	Good	Low
AFLP	All	Good	Good	Moderate	Good	Low to moderate
SDS-PAGE of WCPs	All	Good	Good	Moderate	Good	Moderate

¹ In all cases the ease of interpretation can be increased by applying objective reading and analysis systems.

in a polyphasic approach will provide a suitable solution for reliable identification and characterization of strains.

Indeed, for the identification and characterization of cultivable microflora of dairy products, in addition to phenotypic traits, molecular techniques with different specificity levels have been applied in the last decade. Tables 2.4 to 2.12 summarize the strategies applied for these purposes in research of microorganisms isolated from the dairy environment. Table 2.3 groups the experiences of researchers who contemporaneously considered more than one LAB genus, while Tables 2.4 to 2.12 report research aiming to characterize strains of the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostocs*, *Pediococcus*, *Propionibacterium* and yeasts.

3.1 Strain Typing

The most widely used typing techniques for the characterization of the microflora of dairy products are: RAPD-PCR (and similar techniques, such as REP-PCR, AP-PCR, BOX-PCR); REA-PFGE; AFLP and SDS-PAGE of WCPs. These techniques are used alone or in combination (Tables 2.3 to 2.11).

For randomly fingerprinting characterization (RAPD-PCR and similar techniques), analysis has been made of patterns obtained by using one primer (Morea, et al. 1999; Baruzzi, et al. 2002; Jenkins, et al. 2002; Gobetti, et al. 2002, Poznanski, et al., 2004, Rossetti and Giraffa 2005; Coppola, et al. 2006, Zamfir, et al. 2006; for other references see Tables 2.3 to 2.11), two or more primers in separate reactions (Succi, et al. 2005, Sanchez, et al. 2006; Giraffa, et al. 2004; for other references see Tables 2.3 to 2.11) or two primers in the same reaction (Bouton, et al. 2002; Callon, et al. 2004). Generally, all the above research used thermal PCR conditions with a single annealing temperature. By contrast, others applied two (Gobetti, et al. 2002; Baruzzi, et al. 2002; Baruzzi, et al. 2000) or three (Jenkins, et al. 2002; Cusick and O'Sullivan 2000) cycling conditions with different annealing temperatures. As summarized by Table 2.12, for each group of microorganisms a different primer(s) was used. The exceptions are represented by RAPD-PCR primers M13, Coc, P32, P1A and PC1 that were used to characterize strains of different LAB genera and yeasts. Finally, Rossetti and Giraffa (2005) established a large RAPD-PCR fingerprint database to identify dairy LAB (*Lb. casei*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. helveticus*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. brevis*, *E. faecium*, *E. faecalis*, *St. thermophilus* and *L. lactis*) on the basis of their M13 RAPD-PCR pattern. In particular, the RAPD technique is quite straightforward and quick, and analysis can generally be performed starting from a lysate of a bacterial colony without the need of extensive DNA purification. However, it is well known that RAPD profiles can be sensitive to even modest changes in the reaction conditions, and this can lead to problems of reproducibility, particularly regarding the minor faint bands, which are not always well conserved among replicates of the same sample. Moreover, although the use of RAPD-PCR protocols was efficiently applied

Table 2.3 Molecular Approaches Used for the Identification, Characterization and Monitoring of LAB Isolated from Dairy Products

Source	Methods Applied ^a	Aims ^b	Reference
Different sources	Statistical analysis of RAPD-PCR fingerprints (<i>M13</i>), sp-PCR (Berthier and Ehrlich 1998; Chagnaud, et al.. 2001; Corroler, et al.. 1998; Drake, et al.. 1996a; Dutka-Malen, et al. 1995; Guameri, et al. 2001; Lick, et al. 1996; Tilsala-Timisjarvi and Alatosava 1997; Torriani, et al. 1999).	I, C	Rossetti and Giraffa 2005
Natural Whey Starters	LH-PCR, sp-PCR (Lick, et al. 1996; Torriani, et al. 1999; Tilsala-Timisjarvi and Alatosava 1997; Chagnaud, et al. 2001).	I	Lazzi, et al. 2004
Toma Piemontese Cheese	16S-23S rDNA ISR polymorphism (Jensen, et al. 1993), sp-PCR (Dutka-Malen, et al. 1995; Lick, et al. 1996; Cheng, et al. 1997; Berthier and Ehrlich 1998; Zlotkin, et al. 1998; Ward and Timmins 1999; Ke, et al. 1999; Corroler, et al. 1999), 16S rDNA sequencing.	I	Fortina, et al. 2003
Italian ewe's milk cheeses	Numerical analysis of SDS-PAGE of WCPs fingerprints	I, C	De Angelis, et al. 2001
Caciocavallo cheeses	Numerical analysis of SDS-PAGE of WCPs fingerprints	I, C	Piraino, et al. 2005
Different sources	Artificial neural networks analysis of SDS-PAGE of WCPs fingerprints	I, C	Piraino, et al. 2006
Romanian Dairy products	rep-PCR fingerprinting (<i>REP-PCR</i>), SDS-PAGE of WCPs, 16S rRNA gene sequencing	I, C	Zamfir, et al. 2006
Tenerife cheese	SDS-PAGE of WCPs fingerprints	I, C	Perez, et al. 2000
Different sources	Sau-PCR	I, C	Corich, et al. 2005
Caciocavallo Pugliese cheese	Two step RAPD-PCR (<i>Coc</i>), 16S rDNA partial sequencing (Cocconcelli, et al. 1997)	I, M	Gobbetti, et al. 2002
Canestrato Pugliese Cheese	16S rDNA-RFLP (Jayarao, et al. 1992), 16S rDNA partial sequencing, sp-PCR (Torriani, et al. 2001)	I, M	Aquilanti, et al. 2006

(continued)

Table 2.3 Molecular Approaches Used for the Identification, Characterization and Monitoring of LAB Isolated from Dairy Products (continued)

Source	Methods Applied ^a	Aims ^b	Reference
Fior di Latte Cheese	RAPD-PCR (<i>Primm</i> 239), <i>Sma</i> I REA-PFGE (Moschetti, et al. 1997), 16S-23S rDNA ISR polymorphism (Blaiotta, et al. 2002), sp-PCR (Fortina, et al. 2001; Moschetti, et al. 2000)	I, M	Coppola, et al. 2006
Mozzarella cheese	RAPD-PCR (<i>Coc</i>), 16S rDNA partial sequencing	I, M	Morea, et al. 1999
Nostrano di Primiero Cheese	RAPD-PCR (<i>PC1</i>), 16S rDNA partial sequencing	I, M	Poznanski, et al. 2004
Raw Milk Salers Cheeses	REP-PCR (<i>Rep-IR-Dt</i> plus <i>REP2-D</i>) ge-PCR (Deasy, et al. 2000), sp-PCR (Berthier and Ehrlich 1999; Berthier, et al. 2001; Ward and Timmins 1999)	I, M	Callon, et al. 2004
Scamorza Altamura Cheese	Two step RAPD-PCR (<i>Coc</i>), 16S rDNA partial sequencing	I, M	Baruzzi, et al. 2002
Swiss cheese starter cultures	Triplex AP-PCR (<i>P32</i>), <i>Sma</i> I and <i>Apa</i> I REA-PFGE	C	Jenkins, et al. 2002

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12); sp-PCR, ge-PCR are species- and genus-specific PCR assays, respectively (Table 2.13).

^b identification; C, characterization; M, monitoring.

Table 2.4 Molecular Approaches Used for the Identification, Characterization and Monitoring of Lactobacilli Isolated from Dairy Products

Source	Methods Applied ^a	Aims ^b	Reference
Different sources	multiplex sp-PCR (seven <i>Lactobacillus</i> species)	I, D	Kwon, et al. 2004
Different sources	Box-PCR (<i>BOXAIR</i>)	I, D (LD, ST)	De Urraza, et al. 2000
Parmigiano Reggiano cheese	RAPD-PCR (<i>M13</i> and <i>D8635</i>), sp-PCR (Ward and Timmins 1999)	I, C (LR)	Succi, et al. 2005
Spanish Goat Cheeses	RAPD-PCR (<i>OPL-05</i> and <i>P1</i>)	C	Sanchez, et al. 2005
Different sources	SDS-PAGE of WCPs fingerprints, <i>Not</i> I REA-PFGE, ARDRA-PCR, Ribotyping	C (LD)	Moschetti, et al. 1997
Natural Starter Cultures	RAPD-PCR (<i>D12554</i> and <i>M13</i>), <i>Not</i> I REA-PFGE	C (LDL)	Giraffa, et al. 2004
Different sources	SDS-PAGE of WCPs fingerprints, Ribotyping, <i>Sma</i> I REA-PFGE	C (LH)	Dimitrov, et al. 2005
Natural Whey Starter Cultures	SDS-PAGE of surface proteins fingerprints, IS1201 <i>Eco</i> RI-RFLP (Reinheimer, et al. 1996)	C (LH)	Gatti, el al. 2004
Natural Whey Starter Cultures and Cheeses	MLRT (eight housekeeping loci), and ARDRA-PCR	C (LH)	Borgo, et al. In Press
Different sources	Sequence analysis of S-layer-encoding genes	C (LH)	Gatti, el al. 2005
Different sources	MLST (six housekeeping loci), Ribotyping and 16S-23S rDNA ISR-RFLP	C (LP)	De Las Rivas, et al. 2006
Natural Whey Cultures	RAPD-PCR (<i>Coc</i>), 16S rDNA partial sequencing	C, M	Cocconelli, et al. 1997
Ricotta Forte Cheese	Two step RAPD-PCR (<i>Coc</i>), 16S rDNA partial sequencing, 16S-23S rDNA sequencing (Berthier and Ehrlich 1998), presence of <i>prtP</i> gene (Klijn, et al. 1995)	C, M	Baruzzi, et al. 2000
Manchego cheese	RAPD-PCR (<i>OPL-05</i> and <i>P1</i>)	C, M	Sanchez, et al. 2006
Danbo Cheese	RAPD-PCR (<i>P1</i>) and TTGE (Vasques, et al. 2001)	C, M	Antonsson, et al. 2003
Different sources	RAPD-PCR (<i>Coc</i> , <i>M13</i> and <i>M14</i>) and AFLP	D (LP)	Torriani, et al. 2001a
Fiore Sardo Cheese	sp-PCR (Drake, et al. 1996b; Berthier and Ehrlich 1998; Ward and Timmins 1999)	I, M	Mannu, et al. 2000a
Comté Cheese	RAPD-PCR (<i>BO6</i> , <i>BO10</i>), REP-PCR (REP-IR-DT1 plus REP2-D), <i>Sgr</i> AI and <i>Xho</i> I REA-PFGE, sp-PCR (Drake, et al. 1996b)	I, M	Bouton, et al. 2002
Different sources	PCR detection of bacteriophages	C (LDL)	Zago, et al. 2006
Different sources	Identification and typing techniques	Review	Coeuret, et al. 2003

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12), sp-PCR, species-specific PCR assays (Table 2.13).

^b I, identification; C, characterization; M, monitoring; D, differentiation. In parenthesis: LD, *Lb. delbrueckii*; LDL, *Lb. delbrueckii* subsp. *lactis*; LH, *Lb. helveticus*; LP, *Lb. plantarum*; LR, *Lb. rhamnosus* ST; S, *thermophilus*.

Table 2.5 Molecular Approaches Used for the Identification and Characterization of Streptococci Isolated from Dairy Sources

Methods Applied ^a	Aims ^b	Reference
Sequence analysis of <i>dnaJ</i> and <i>gyrB</i> genes	I, D	Itoh, et al. 2006
Sequence analysis of 16S-23S rDNA ITS, multiplex ITS-SSCP	I, D	Mora, et al. 2003
16S-23S rDNA ITS patterns, RAPD-PCR (<i>XD8</i> and <i>XD9</i>), <i>Sma</i> I REA-PFGE	I, D, C (EN also)	Moschetti, et al. 1998
RAPD-PCR (<i>D11344</i> and <i>M13</i>) and sp-PCR (this study)	I, D (SM)	Lombardi, et al. 2004
RAPD-PCR (<i>D11344</i> and <i>M13</i>) and sp-PCR (Lick, et al. 1996)	C (ST)	Andrighetto, et al. 2002
RAPD-PCR (<i>XD9</i>) and <i>galR-galK</i> intergenic region sequence analysis	C (ST)	de Vin, et al. 2005
Sequences analysis of the <i>lacSZ</i> operon, group-specific PCR systems (strain-specific PCR, <i>lacS</i> -PCR-SSCP, <i>lacS</i> -PCR-DGGE)	C (ST)	Ercolini, et al. 2005
RAPD- PCR (<i>M13</i>) and sp-PCR (Lick, et al. 1996)	C (ST)	Giraffa, et al. 2001
sp-PCR (Lick, et al. 1996), RAPD- PCR (<i>OP1-02mod</i> , <i>XD9</i>), REA of <i>epsC-D</i> locus	C (ST)	Mora, et al. 2002a
<i>Sma</i> I, <i>Sfi</i> I, <i>Bss</i> HII and <i>Not</i> I REA-PFGE	C (ST)	O'Sullivan and Fitzgerald 1998

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

^b I, identification; C, characterization; D, differentiation. In parenthesis: ST, *S. thermophilus*; SM, *S. macedonicus*; EN, enterococci.

Table 2.6 Molecular Approaches Used for the Identification, Characterization and Monitoring of Lactococci Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
Different sources	RAPD-PCR (<i>P1A</i> , <i>P2C</i> and <i>P3A</i>)	C (LL)	Taillez, et al. 1998
Pecorino Sardo Cheese	sp-PCR (Garde, et al. 1999), Plasmid profile, <i>Sma</i> I REA-PFGE	I, C (LL)	Mannu, et al. 2000b
Different sources	sp-PCR (Nomura, et al. 2002), triplex RAPD-PCR (<i>P1A</i> , <i>P2A</i> and <i>P3A</i>), plasmid profile	I, C (LL)	Nomura, et al. 2006
Fior di Latte Cheese	16S-23S rDNA ITS patterns	I, D	Blaiotta, et al. 2002
Spain Starter-free Cheeses	RAPD-PCR (<i>RAP3</i>) <i>Mbo</i> II and <i>Hha</i> I ARDRA patterns, <i>Apa</i> I or <i>Sma</i> I REA-PFGE, SDS-PAGE of WCPs fingerprints	I, D (EN also)	Delgado and Mayo 2004
Fior di Latte Cheese	multiplex PCR (16S-23S rDNA ITS/ <i>nisA</i> gene), <i>Sma</i> I REA-PFGE	M (LL <i>nis+</i>)	Moschetti, et al. 2001
Pecorino Sardo Cheese	sp-PCR (Ke, et al. 1999; Mannu, et al. 2000b), Plasmid profile, <i>Sma</i> I REA-PFGE	M (LL, EN)	Mannu and Paba 2002

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

^b I, identification; C, characterization; D, differentiation; M, monitoring. In parenthesis: LL, *L. lactis*; EN, enterococci.

Table 2.7 Molecular Approaches Used for the Identification, Characterization and Monitoring of Enterococci Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
Portuguese cheese	RAPD-PCR (<i>M13</i> and <i>D8635</i>), proteins profile, fatty acids profile	C	Silva, et al. 2003
Different sources	Detection of virulence and vancomycin resistance gene markers by PCR	C	Khan, et al. 2005
Northwest Italian Dairy Products	RAPD-PCR (<i>M13</i> and <i>D8635</i>), sp-PCR (Dutka-Malen, et al. 1995)	I, C	Morandi, et al. 2006
Bryndza Cheese	sp-PCR (Dutka-Malen, et al. 1995; Knijff, et al. 2001), Detection of virulence genes by PCR (Dupre, et al. 2003; Jurkovic, et al. 2006)	I, C	Jurkovic, et al. 2006
Batzos Cheese	RAPD-PCR (<i>M13</i> and <i>D8635</i>), sp-PCR (Dutka-Malen, et al. 1995), <i>Sma</i> I REA-PFGE, plasmid profile	I, C	Psoni, et al. 2006
Fiore Sardo Cheese	RAPD- PCR (<i>XD9</i> and <i>M13</i>)	I, C.	Cosentino, et al. 2004
Artisanal Italian Cheeses	16S rDNA sequencing, DNA-DNA hybridization, 16S-23S rDNA ITS pattern	I, D	Fortina, et al. 2004
Different sources	MLSA (sequence analysis of <i>rpoA</i> , <i>pheS</i> and <i>atpA</i> genes)	I, D	Naser, et al. 2005a,b
Semicotto Caprino Cheese	RAPD-PCR (<i>M13</i> and <i>D8635</i>)	I, M	Suzzi, et al. 2000
Cheddar-type Cheese	RAPD-PCR (<i>D11344</i>), <i>Sma</i> I REA-PFGE	I, M	Gelsomino, et al. 2002
Different sources	Identification and typing techniques	Review	Domig, et al. 2003

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

^b I, identification; C, characterization; D, differentiation; M, monitoring.

Table 2.8 Molecular Approaches Used for the Identification and Characterization of *Leuconostocs* Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
Different sources	multiplex sp-PCR (5 species)	I, D	Lee, et al. 2000
Different sources	Protein profile, Ribotyping, ARDRA-PCR, <i>Apa</i> I REA-PFGE	I, D	Villani, et al. 1997
Different sources	RAPD-PCR (<i>Primm</i> 239), sp-PCR (this study)	I (LM)	Moschetti, et al. 2000
French Cheeses	RAPD-PCR (<i>P1A</i> and <i>P3A</i>), 16S rDNA sequencing, sp-PCR (this study)	C (LM, LCT)	Cibik, et al. 2000

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2-12). sp-PCR, species-specific PCR assays (Table 2-13).

^b I, identification; C, characterization; D, differentiation. In parenthesis: LM, *Ln. mesenteroides*; LCT, *Leuconostoc citreum*.

Table 2.9 Molecular Approaches Used for the Identification and Characterization of *Pediococci* Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
No dairy origin	RAPD-PCR (<i>P1</i>)	I	Nigatu, et al. 1998
Different sources	Multilocus Hybridization Typing (16S rDNA, <i>rpoC</i> , <i>ldhD</i> , <i>ldhL</i> , and <i>metS</i> probes)	C (PAC)	Mora, et al. 2002b
Different sources	RAPD-PCR (<i>P1</i> and <i>P2</i>), <i>Apa</i> I, <i>Sma</i> I, <i>Asc</i> I, <i>Not</i> I, or <i>Sfi</i> I REA-PFGE	I, C	Simpson, et al. 2002

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12).

^b I, identification; C, characterization. In parenthesis: PAC, *P. acidilactici*.

by Rossetti and Giraffa (2005), for LAB identification the 16S rRNA gene sequencing for some strains was needed.

RAPD-PCR and similar techniques may have the advantage of facilitating simultaneous strain typing, species affiliation determination and individual strain differentiation. Application of these techniques for analyzing isolates from the most important steps of cheese fermentation allowed monitoring of LAB species and biotypes within species during cheese manufacturing and ripening, and the detection of dominant biotypes in each fermentation phase (for references see Tables 2.3 to 2.11).

Morea, et al. (1999) monitored LAB during manufacture of Mozzarella cheese. Of the 25 RAPD-PCR biotypes found, only one (referable to *St. thermophilus* species) was detected in all samples analyzed (whey, curd, Mozzarella after shaping and after 24 hours of storage). Two other biotypes (referable *Enterococcus* spp. and *L. lactis* species, respectively) were found in curd and cheese samples. Coppola, et al. (2006) analyzed RAPD-PCR patterns of *L. lactis* strains from raw milk, curd and

Table 2.10 Molecular Approaches Used for the Identification and Characterization of *Propionibacteria* Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
Dairy	Partial Least Squares (PLS) regression analysis of RAPD-PCR patterns (<i>P1B</i> and <i>P2B</i>), 16S rDNA sequencing	I, C	Matte-Taillez, et al. 2002
Different sources	RAPD-PCR (<i>OPL-01</i> , <i>OPL-02</i> and <i>OPL-5</i>) and <i>Sma</i> I CGE-REA profiles analysis	I, C	Rossi, et al. 1998
Different sources	sp-PCR (this study)	I, D	Rossi, et al. 1999
Dairy	<i>recA</i> gene sequence analysis	I, D	Rossi, et al. 2006
Swiss cheese starter cultures	Triplex AP-PCR (<i>P32</i>), <i>SpeI</i> and <i>XbaI</i> REA-PFGE	C	Jenkins, et al. 2002

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12). sp-PCR, species-specific PCR assays (Table 2.13).

^b I, identification; C, characterization; D, differentiation.

Table 2.11 Molecular Approaches Used for the Identification and Characterization of Yeasts Isolated from Dairy Sources

Source	Methods Applied ^a	Aims ^b	Reference
Italian Dairy Products	RAPD-PCR (<i>M13</i> and <i>RF2</i>)	I, D	Andrighetto, et al. (2000)
Hungarian dairy products	18S rDNA/ITS1-RFLP, RAPD-PCR (<i>M13</i>)	I, D	Vasdinyei and Deak (2003)
Sardinian ewe's Dairy Products	no molecular techniques	OC	Cosentino, et al. (2001)
Raw Milk Salers Cheeses	26S rDNA gene sequencing, SSCP (of V4 region of 18S rDNA)	I	Callon, et al. (2006)
Smear-Ripened Cheeses	Mitochondrial DNA restriction fragment length polymorphism (mtDNA-RFLP), Fourier-transform infrared spectroscopy (FTIR)	I, C	Mounier, et al. (2005, 2006)

^a The name of RAPD-PCR primer used is italicized (the sequence of the primer is reported in Table 2.12).

^b I, identification; C, characterization; D, differentiation; OC, only the occurrence was evaluated.

Fior di latte cheese, and showed that five out of eight biotypes isolated from raw milk also persist during curd ripening. Moreover, isolates showing one unique RAPD-PCR pattern were detected in all the monitored phases. The results underlined the importance of raw milk as a source of important bacteria for fermentation. Moreover, statistical analysis of RAPD-PCR results can allow grouping of strains on the basis of their geographical and dairy origin (Moschetti, et al. 1998). RAPD-derived probes and primers have been described for identification at species level, and even at strain level (Quere, et al. 1997; Erlandson and Bat 1997).

Table 2.12 The Most Used RAPD-PCR Primers for the Characterization of Dairy Microorganisms

Name ^a	Sequence (5'—3')	Used for characterization of	Used by
BOXAIR	CTACGGCAAGCGACGCTGACG	<i>Lactobacillus</i> <i>Streptococcus</i>	Versalovic, et al. 1994; De Urraza, et al. 2000
Coc	AGCAGCGTGG	LAB <i>Lactobacillus</i>	Cocconcelli, et al. 1995; Cocconcelli, et al. 1997; Morea, et al. 1999; Baruzzi, et al. 2000; Baruzzi, et al. 2002; Torriani, et al. 2001a; Gobbetti, et al. 2002
D11344	AGTGAATTCGCGGTGAGATGCCA	<i>Streptococcus</i> <i>Enterococcus</i>	Akopyanz, et al. 1992; Gelsomino, et al. 2001; Gelsomino, et al. 2002; Andrighetto, et al. 2002; Lombardi, et al. 2004
D1254	CCGCAGCCAA	<i>Lactobacillus</i>	Akopyanz, et al. 1992; Giraffa, et al. 2004
D8635	GAGCGGCCAAAGGGAGCAGAC	<i>Lactobacillus</i> <i>Enterococcus</i>	Akopyanz, et al. 1992; Suzzi, et al. 2000; Silva, et al. 2003; Succi, et al. 2005; Morandi, et al. 2006; Psoni, et al. 2006
M13	GAGGGTGGCGGTTCT	LAB <i>Lactobacillus</i> <i>Streptococcus</i> <i>Enterococcus</i> <i>Yeasts</i>	Huey and Hall 1989; Suzzi, et al. 2000; Andrighetto, et al. 2000; Giraffa, et al. 2001; Torriani, et al. 2001a; Andrighetto, et al. 2002; Silva, et al. 2003; Vasdinyei and Deak 2003; Cosenino, et al. 2004; Lombardi, et al. 2004; Giraffa, et al. 2004; Rossetti and Giraffa 2005; Succi, et al. 2005; Morandi, et al. 2006; Psoni, et al. 2006
M14	GAGGGTGGGGCCGTT	<i>Lactobacillus</i>	Torriani, et al. 2001a
OPL-02mod	GCCTGGAGGAGAGG	<i>Streptococcus</i>	Mora, et al. 2002a
OPL-01	GGCATGACCT	<i>Propionibacterium</i>	Rossi, et al. 1998
OPL-02	TGGGCGTCCAA	<i>Propionibacterium</i>	Rossi, et al. 1998
OPL-05	ACGCAGGCA	<i>Lactobacillus</i>	Sanchez, et al. 2005; Sanchez, et al. 2006
OPL-05A	ACGCAGGCAC	<i>Propionibacterium</i>	Rossi, et al. 1998
P1 (9mer, LPL)	ACGCGCCCT	<i>Lactobacillus</i> <i>Pediococcus</i>	Johansson, et al. 1995; Nigatu, et al. 1998; Fitzsimons, et al. 1999; Antonsson, et al. 2003; Simpson, et al. 2002; Sanchez, et al. 2005; Sanchez, et al. 2006
P1A (RAP3, B06)	TGCTCTGCCC	<i>Lactobacillus</i> <i>Lactococcus</i> <i>Enterococcus</i>	Tailliez, et al. 1998; Mangin, et al. 1999; Cibik, et al. 2000; Bouton, et al. 2002; Delgado and Mayo 2004; Nomura, et al. 2006
P1B	CGGCCTGGAC	<i>Propionibacterium</i>	Matte-Tailliez, et al. 2002

(continued)

Table 2.12 The Most Used RAPD-PCR Primers for the Characterization of Dairy Microorganisms (continued)

Name ^a	Sequence (5'—3')	Used for characterization of	Used by
P2	ATGTAACGCC	<i>Pediococcus</i>	Fitzsimons, et al. 1999; Simpson, et al. 2002
P2A	GGTGACGCAG	<i>Lactococcus</i>	Mangin, et al. 1999; Nomura, et al. 2006
P2B	CGCCTGCCC	<i>Propionibacterium</i>	Matte-Tailliez, et al. 2002
P2C	GTGACGCAG	<i>Lactococcus</i>	Tailliez, et al. 1998
P32	CAGACGCCGGTAATWC	LAB	Cusick and O'Sullivan 2000; Jenkins, et al. 2002
P3A (BO10)	CTGCTGGGAC	<i>Lactobacillus</i>	Tailliez, et al. 1998; Mangin, et al. 1999; Cibik, et al. 2000; Bouton, et al. 2002; Nomura, et al. 2006
		<i>Lactococcus</i>	
		<i>Leuconostoc</i>	
PC1	AGCAGGCTCG	LAB	Poznanski, et al. 2004
Primm 239	CTGAAGCGGA	<i>Leuconostoc</i>	Moschetti, et al. 2000; Coppola, et al. 2006
		<i>Lactococcus</i>	
REP-IR-Dt	NCGNCGNCATCNGGC	LAB	Versalovic, et al. 1991; Callon, et al. 2004
REP-IR-DT1	IIINCNCGNCATCNGGC	<i>Lactobacillus</i>	Bouton, et al. 2002
REP2-D	NCGNCCTATCNGGCCTAC	LAB	Versalovic, et al. 1991; Callon, et al. 2004; Bouton, et al. 2002
		<i>Lactobacillus</i>	
REP-PCR	GTGGTGGTGGTGGTG	LAB	Zamfir, et al. 2006
RF2	CGGCCCTGT	Yeast	Paffetti, et al. 1995; Andrighetto, et al. 2000
RP	CAGCACCCAC		Ward and Timmins 1999
		<i>Lactobacillus</i>	
XD8	CAAGGCATCC	<i>Streptococcus</i>	Moschetti, et al. 1998
		<i>Enterococcus</i>	
XD9	GAAGTCGTCC	<i>Streptococcus</i>	Moschetti, et al. 1998; Mora, et al. 2002a; Cosentino, et al. 2004; de Vin, et al. 2005
		<i>Enterococcus</i>	

^a Some primers were grouped (different authors named primers showing the same sequence with different names) some others were renamed (primers with different sequences were named with same name).

REA-PFGE, performed by rare cutting endonucleases, has also been widely applied to type LAB isolates. The choice to use the endonuclease is of crucial importance to obtain reliable differentiation of the isolates. Endonuclease *Sma* I was used to type streptococci (Moschetti, et al. 1997; O'Sullivan and Fitzgerald 1998), enterococci (Gelsomino, et al. 2001; Psoni, et al. 2006), *L. lactis* (Moschetti, et al. 2001; Mannu, et al. 2000b; Delgado and Mayo 2004) and *Lb. helveticus* (Coppola, et al. 2006), *Apa* I for typing *Ln. mesenteroides* (Villani, et al. 1997), *Not* I for typing *Lb. delbrueckii* (Moschetti, et al. 1997; Giraffa, et al. 2004) and *Pediococcus* spp. (Simpson, et al. 2002). In some cases more than one endonuclease was used. Patterns obtained by *Sma* I and *Apa* I were analyzed by Jenkins, et al. (2002) to differentiate Swiss cheese starter culture strains of *Lb. helveticus*, *St. thermophilus* and *Prop. Freudenreichii*, and by Delgado and Myo (2004) to evaluate genetic diversities of *Lc. lactis* and *Enterococcus* spp. isolated from Spanish starter-free cheeses. *Sgr* AI and *Xho* I were applied by Bouton, et al. (2002) to monitor *Lb. helveticus* and *Lb. delbrueckii* subsp *lactis* strains isolated during Comté cheese ripening. Simpson, et al. (2002) evaluated the discrimination power of different endonucleases (*Apa* I, *Sma* I, *Asc* I, *Not* I, *Sfi* I) for differentiation of *Pediococcus* spp. strains. REA-PFGE, albeit a laborious and expensive method, is highly reproducible and is, therefore, considered to offer the highest resolution for strain differentiation of LAB. Generally, analysis of RAE-PFGE patterns obtained by one well-chosen enzyme can provide fine, reliable differentiation. However, it has been suggested that analysis of two or three restriction enzymes should be used to differentiate *Lactobacillus* strains (Vancanneyt, et al. 2006c).

Blaiota, et al. (2001) used REA-PFGE to monitor the addition of LAB, used as starter, to Cacioricotta cheese. By analyzing isolates from different phases of the fermentation the technique made it possible to evaluate the growth kinetics of each starter strain during the process.

Bouton, et al. (2002) used fingerprinting PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. Isolates, which exhibited unique patterns by RAPD or REP-PCR, were distinguishable by PFGE. By contrast, some strains which were distinguishable by RAPD or REP-PCR were related by PFGE. These discrepancies were explained by the different exploration of DNA polymorphism (the whole DNA chromosome for PFGE, and region amplified by primers for RAPD and the REP-PCR). The use of second restriction enzymes would certainly be useful in this case.

Jenkins, et al. (2002), in analyzing genetic diversity in Swiss cheese starter cultures, found that strains with > 87 percent similarity by REA-PFGE consistently had the same acidification rate.

As it is a time-consuming technique, REA-PFGE was applied when fine strain typing was needed, when a small number of isolates have to be typed and when other strain typing techniques may be unreliable. Therefore, in many cases, it is applied as a supplementary technique to confirm or improve results obtained by other typing methods. Moschetti, et al. (1997) analyzed *Not* I-REA-PFGE patterns of *Lb. delbrueckii* subsp. *bulgaricus* isolated from commercial yogurt and showed that some strains isolated from products of different dairies displayed the same pattern,

suggesting that different dairies used the same starter. Similar results were obtained by Vancanneyt, et al. (2006c) who applied REA-PFGE to confirm and/or improve results obtained by AFLP analyzing *Lb. rhamnosus* strains isolated from different commercial probiotic preparation. Coppola, et al. (2006) analyzed *Lb. helveticus* strains isolated during manufacture of fior di latte cheese by RAPD-PCR and *Sma*I-REA-PFGE. Of 55 strains only four RAPD-PCR profiles were found by using primer Primm 239 (reliably used to differentiate *Lc. lactis* strains). Therefore, for a more appropriate biotyping, *Sma*I-REA-PFGE was applied. Using this last technique, a total of 13 different patterns were found. Also in this case, as already shown in *Lc. lactis* strains, strains showing the same profile were found in milk, in curd at the beginning of ripening and in curd at the end of ripening.

Overall, the most reliable method for strain differentiation is still REA-PFGE analysis and, therefore, its application is going to be fundamental for the monitoring of microorganisms in dairy processing.

The AFLP technique was used only to differentiate and characterize some species of the *Lb. plantarum* group (*Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum*) by Torriani, et al. (2001a). Fluorescent AFLP (FAFLP) was also applied to type probiotic *Lb. rhamnosus* strains by Vancanneyt, et al. (2006c). The AFLP technique normally displays good levels of reproducibility and reliability – apart from some reported problems related to the initial DNA concentration or to the endonuclease or ligase treatment efficiency – but it is quite laborious and time-consuming, given that it requires two enzymatic reactions and large polyacrylamide gels to reach a good level of band separation. Although the observed strain-to-strain variations in the FAFLP patterns within a given cluster may reflect strain-specific differences, such variations are, in most cases, introduced during data processing. Therefore, for strain typing, FAFLP should be complemented by other fingerprinting techniques such as PFGE (Vancanneyt, et al. 2006c). However, FAFLP performed by multiple primer combination has proved to be a valid and powerful tool to reveal intraspecies diversities (Vancanneyt, et al. 2002). Recently, a simplified AFLP technique, called Sau-PCR, was applied to LAB fingerprinting (Corich, et al. 2005). Results suggest that Sau-PCR may be considered for DNA fingerprinting based on analyses as a possible alternative to the RAPD technique in cases where reproducibility or polymorphism levels are not satisfactory, and as an alternative to the AFLP technique, but with lower costs in terms of time and equipment, when a restriction-plus-amplification approach is preferred. However, AFLP, FAFLP and Sau-PCR were never used for typing large numbers of isolates from the dairy environment.

SDS-PAGE of WCPs was also applied to characterize cultivable dairy microflora. Villani, et al. (1997) evaluated diversities of *Ln. mesenteroides* strains isolated from dairy and non-dairy environments; Moschetti, et al. (1997) of *Lb. delbrueckii* isolated from yogurt, raw and pasteurized milks; Rossi, et al. (1998) propionibacteria from different dairy sources; Silva, et al. (2003) isolated enterococci from an artisanal Portuguese cheese, and Delgado and Mayo (2004) isolated lactococci and enterococci from Spanish starter-free cheeses. Piraino, et al. (2005) and Zamfir, et al. (2006) applied SDS-PAGE of WCPs to identify and characterize LAB occurring

in caciocavallo cheeses and Romanian dairy products, respectively. Finally, Piraino, et al. (2006) applied unsupervised and supervised artificial neural networks for the identification of LAB (*Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Lactococcus* and *Streptococcus*) on the basis of their SDS-PAGE of the WCP pattern. SDS-PAGE of surface proteins was applied by Gatti, et al. (2004) to differentiate *Lb. helveticus* strains isolated from different natural whey starter cultures. However, there is some evidence of poor differentiation of some LAB species by this technique. De Angelis, et al. (2001), analyzing LAB of 12 Italian ewe's milk cheeses, showed that strains of *Lb. plantarum* and *Lb. pentosus* grouped in the same cluster. Gancheva, et al. (1999), analyzing a set of 98 strains belonging to nine species of the *Lb. acidiphilus* group had difficulty differentiating between *L. johnsonii* and *Lb. gasseri* strains, and between those of *Lb. gallinarum* and *Lb. amylovorus*.

However, statistical analysis of SDS-PAGE of WCPs provides an effective tool for the classification and identification of LAB (Piraino, et al 2005 and 2006). By applying this technique Piraino, et al. (2005) demonstrated the possibility of discriminating PDO cheeses from non-PDO and showed that the microflora of PDO cheeses was less heterogeneous than that of non-PDO cheeses, and consisted mainly of non-starter LAB. Finally, in some cases the discrimination power of this technique was comparable to that of REA-PFGE (Delgado and Mayo 2004).

In addition to the above typing options, some authors have developed assays targeting genes encoding for key proteins or enzymes in food-borne bacteria. Gatti, et al. (2005) evaluated diversities of surface layer (S-layer) protein genes in *Lb. helveticus* strains and demonstrated that heterogeneity exists in genes of this species. However, cluster analysis of the sequences separated strains into only two main clusters. Ercolini, et al. (2005) evaluated sequence diversities of *lacZS* operon of dairy *St. thermophilus* strains. Due to sequence polymorphism it was possible to design PCR-DGGE and PCR-SSCP systems allowing four and two groups, respectively, to be detected among strains analyzed. Moreover, a specific PCR system allowing detection of only one group of strains was designed. De Vin, et al. (2005), analyzing *galR-galK* (regulator and galactokinase genes, respectively) intergenic region of 49 *St. thermophilus* strains, found eight different genotypes. Of the latter, only four were related to the Gal-positive phenotype.

MLST (multi-locus sequence typing), which exploits the genetic variation present in six housekeeping loci, was recently applied to determine the genetic relationship among *Lb. plantarum* isolates (De Las Rivas, et al. 2006). Of the 16 strains analyzed, there were 14 different allelic combinations, with 12 of them represented by only one strain. MLHT (multi-locus hybridization typing) performed by five housekeeping gene probes was used by Mora, et al. (2002b) to subgroup *P. acidilactici* strains. MLRT (multi-locus restriction typing) analyzing restriction patterns from eight loci of housekeeping genes was applied by Borgo, et al. (2007) to characterize *Lb. helveticus* strains isolated from whey starter cultures and cheeses. High heterogeneity among strains was shown and an excellent association was observed between restriction profiles and origin of most of the isolates analyzed.

These last typing or sub-grouping approaches (Gatti, et al. 2005; Ercolini, et al. 2005; De Las Rivas, et al. 2006; Mora, et al. 2002b) have not yet been applied to

characterize or monitor wild strains isolated from dairy ecosystems. However, these techniques have reached a great level of automation and will surely have an important role in the rapid typing of bacteria of dairy origin.

Ribotyping was applied to evaluate genetic diversity of *Leuconostoc* spp. (Villani, et al. 1997), to differentiate *Lb. delbrueckii* subspecies (Moschetti, et al. 1997), to differentiate and characterize strains of *Lb. casei* group species (Svec, et al. 2005) and to subgroup *Lb. plantarum* strains (De Las Rivas, et al. 2006). Originally, ribotyping was intended for taxonomic use (Grimont and Grimont 1986), but it was also later applied for typing strains. However, due to its weak discriminatory power as a typing method, other techniques have replaced it. With a commercially available system, all stages of manual ribotyping can be performed and the basic protocol takes at least five days. However, the development of an automated ribotyping system, the RiboPrinter[®], (Qualicon Inc., Wilmington, Del., U.S.) made it possible to shorten the procedure to eight hours. The process is highly standardized and data are stored electronically. In addition, data can be exchanged between different laboratories. Using more than one enzyme, the RiboPrinter[®] proved to be a valuable primary typing method for pathogens (Grif, et al. 2003). Research performed by Brunner, et al. (2000) provides evidence that PFGE and automated ribotyping are two reliable methods that can be useful for epidemiologic investigations on group A streptococci. Most strains belonging to the *Lb. casei* group and the *Lb. acidophilus* group were discriminated at the species level by automated ribotyping (Chun, et al. 2001). Massi, et al. (2004) compared automated ribopatterns of seven probiotic *Lactobacillus* strains (*Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. casei*, *Lb. plantarum*, *Lb. brevis*, *Lb. salivarius* subsp. *salicinius*, *Lb. gasseri*) with those reported in the RiboPrinter[®] database. All probiotic *Lactobacillus* strains gave specific new fingerprinting patterns, as none of them was included in the pre-existing ribogroups of the RiboPrinter[®] database. Due to the ribotyping specificity, the authors concluded that the method represents a powerful tool for strain-specific detection of these lactobacilli. However, Kitahara, et al. (2005), analyzing automated ribopatterns of 22 *Lb. sanfranciscensis* strains, obtained only four clusters at less than 80 percent similarity, while Basaran, et al. (2001) obtained only 10 different ribopatterns analyzing 20 lactococci. However, cluster analysis of data allowed differentiation of *Lc. lactis* subsp. *cremoris* strains from those of *Lc. lactis* subsp. *lactis*. Beasley and Saris (2004) applied RiboPrinter[®] technology to differentiate nisin producing *Lc. lactis* strains isolated from human and cow's milk.

3.2 Identification at Genus, Species and Subspecies Level

Species identification can be achieved by statistical analysis of fingerprint data obtained from the above described approaches, even if they are commonly used for strain typing. Moreover, other techniques can be used to achieve identification at species level.

PCR-RFLP of the amplified 16S rRNA gene (ARDRA-PCR) or 16S-23S rRNA intergenic spacer region (ISR-RFLP-PCR) was also applied to identify or characterize dairy LAB. Villani, et al. (1997) analyzed *Eco RI* and *Hind III* ARDRA-PCR patterns of *Leuconostoc* spp. and showed that the technique is unreliable for species differentiation. By contrast, Aquilanti, et al. (2006) identified enterococci by analyzing *MboII*, *MspI* and *RsaI* PCR-ARDRA patterns. De Las Rivas, et al. (2006) found inconsistent differences on analyzing the ISR-RFLP-PCR of *Lb. plantarum* strains. Indeed, the latter approach was proposed to identify and differentiate *Lactobacillus* species (Moreira, et al. 2005). Baruzzi, et al. (2000) differentiated *Lb. planatrum* and *Lb. paraplantarum* after sequence analysis of the 16S-23S rDNA ISR. Flint and Angert (2006), on the basis of the 16S-23S rDNA ISR sequence, designed a strain-specific PCR primer to identify and monitor *Lactobacillus* spp. HOFG1 (closely related *L. animalis* or *L. murinus* species) in cattle feed.

16S-23S rDNA ISR pattern analysis allows differentiation of dairy streptococci, enterococci and lactococci (Moschetti, et al. 1998; Blaiotta, et al. 2002; Fortina, et al. 2003; Mora, et al. 2003) while it is unreliable for identification and differentiation of lactobacilli.

However, the continuously accumulating set of fingerprinting data and the construction of reliable databases require a high degree of standardization in experimental methodology. It is also important to have high-performing bioinformatic tools at one's disposal to get the best possible information from huge quantities of fingerprinting data. The development of bioinformatics has enabled improvement of the interpretation and elaboration of microbiological data. Many bioinformatic software programs or on-line tools, which are often commercially available, enable nucleic acid (or protein) sequences, fingerprinting profiles and phenotypic data to be analyzed and integrated. Some computerized databases of LAB fingerprints are also available, such as the RFLP database of total DNA patterns (Chan, et al. 2003), SDS-PAGE protein databases (Pot, et al. 1994; Leisner, et al. 1999) and the commercial RiboPrinter® system (Dawson 2001). Acquisition of specialized programs, which are expensive and demand a high level of technical skill for their efficient use, is necessary so that the most important international microbial collections can manage, compare and implement databases with information on genotypic and phenotypic data (Rossetti and Giraffa 2005).

For reliable identification of strains, partial or full 16S rRNA gene sequences have been extensively compared (see Tables 2.3 to 2.11). However, the 16S rRNA gene shows discrimination pitfalls in the identification of closely related LAB species. The genes present in only one copy, such as the Elongation factor Tu (*tuf*) gene (Chavagnat, et al. 2002; Ventura, et al. 2003), the DNA repair recombinase (*recA*) (Felis and Dellaglio 2005), the chaperonin Hsp60 (*Cpn60*) (Dobson, et al. 2004) and the RNA polymerase B subunit (*rpoB*) (Rantsiou, et al. 2004), have been exploited for the differentiation of *Lactobacillus* species. These genes have significant advantages over the 16S rRNA gene because of their species-discrimination power, indicated by published studies to be one order of magnitude higher than that of the 16S rRNA gene (Ventura, et al. 2003). Itoh, et al. (2006) performed sequence analysis of *dnaJ* (a member of the Hsp70 protein family) and *gyrB* (the B-subunit

of DNA gyrase, topoisomerase type II) genes of streptococci and concluded that they are efficient alternative targets for the classification of the genus *Streptococcus*, and that *dnaJ* is suitable for phylogenetic analysis of closely related *Streptococcus* strains. Goh, et al. (2000) sequenced a 552-bp region of the chaperonin 60 gene (*Cpn60*) and demonstrated that clustering of the analyzed species is similar to the published *Enterococcus* trees based on 16S rRNA gene sequences. Poyart, et al. (2000) partially sequenced the gene encoding manganese-dependent superoxide dismutase (*sodA*) in 19 enterococcal-type strains. Their results confirm that the *sodA* gene constitutes a more discriminative target sequence than the 16S rRNA gene and allows differentiation among closely related bacterial species. Rossi, et al. (2006) suggest that the *recA* gene can be used as an alternative to the 16S rRNA gene as a target for detecting/identifying propionibacteria species, but it is less reliable as a molecular marker for their classification and intraspecies distinction.

Species-specific single or multiplex PCR assays were designed and used for rapid identification of LAB occurring in dairy products (Tables 2.3 to 2.11). As reported in Table 2.13, specific PCR systems are now available for the most important bacterial species occurring in dairy products. Moreover, PCR was also used to detect specific genes encoding for particular traits. Some of these systems are: detection of the *prtP* gene (coding for a cell envelope proteinase in LAB) (Klijn, et al. 1995); detection of virulence or resistance factors in enterococci (Khan, et al. 2005; Domig, et al. 2003); detection of genes involved in the production of biologically active amines such as histamine, tyramine and putrescine in LAB (Fernandez, et al. 2004; Marcobal, et al. 2005; Aymerich, et al. 2006).

Table 2.13 Some Available Genus- and Species-specific PCR Assays for the Rapid Identification and/or Differentiation Dairy Microorganisms

Genera	Level of specificity	References
<i>Lactobacillus</i> (<i>Lb.</i>)	<i>Lb. plantarum</i> group species differentiation	Torriani, et al. 2001
	<i>Lb. helveticus</i>	Fortina, et al. 2001
	Differentiation of <i>Lb. casei</i> group species	Ward and Timmins 1999
	<i>Lb. plantarum</i> , <i>Lb. curvatus</i> and <i>Lb. sakei</i>	Berthier and Ehrlich 1998
	<i>Lb. acidophilus</i> , <i>Lb. delbrueckii</i> , <i>Lb. casei</i> , <i>Lb. gasseri</i> , <i>Lb. plantarum</i> , <i>Lb. reuteri</i> and <i>Lb. rhamnosus</i>	Kwon, et al. 2004
	<i>Lb. delbrueckii</i> subsp. <i>lactis</i> and <i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i>	Torriani, et al. 1999
	<i>Lb. helveticus</i> , <i>Lb. paracasei</i> and <i>Lb. rhamnosus</i>	Tilsala-Timisjarvi and Alatossava 1997
	<i>Lb. fermentum</i> , <i>Lb. casei/paracasei</i> , <i>Lb.</i> <i>plantarum</i> , <i>Lb. reuteri</i> and <i>Lb. salivarius</i>	Chagnaud, et al. 2001
	<i>Lb. acidophilus</i> , <i>Lb. casei</i> , <i>Lb. brevis</i>	Massi, et al. 2004
	<i>Lb. brevis</i>	Guarneri, et al. 2001
	<i>Lb. casei</i> , <i>Lb. delbrueckii</i> and <i>Lb. helveticus/acidophilus</i> groups	Drake, et al. 1996a-b

(continued)

Table 2.13 Some Available Genus- and Species-specific PCR Assays for the Rapid Identification and/or Differentiation Dairy Microorganisms (continued)

Genera	Level of specificity	References
<i>Streptococcus</i> (S.)	<i>S. thermophilus</i>	Lick, et al. 1996
	<i>S. macedonicus</i>	Papadelli, et al. 2003
	<i>S. macedonicus</i>	Lombardi, et al. 2004
<i>Enterococcus</i> (E.)	<i>Enterococcus</i> spp.	Deasy, et al. 2000
	<i>E. faecium</i> and <i>E. faecalis</i>	Dutka-Malen, et al. 1995
	<i>Enterococcus</i> spp.	Ke, et al. 1999
	<i>E. faecium</i>	Cheng, et al. 1997
	<i>E. durans</i> and <i>E. hirae</i>	Knijff, et al. 2001
<i>Lactococcus</i> (Lc.)	<i>Lactococcus</i> spp.	Deasy, et al. 2000
	<i>Lc. lactis</i> subsp. <i>lactis</i> and <i>Lc. lactis</i> subsp. <i>cremoris</i>	Corroler, et al. 1998
	<i>Lc. lactis</i> (histidine biosynthesis operon)	Corroler, et al. 1999
	<i>Lc. garvieae</i>	Zlotkin, et al. 1998
	<i>Lc. lactis</i>	Mannu, et al. 2000b
	<i>Lc. lactis</i> subsp. <i>lactis</i> and <i>Lc. lactis</i> subsp. <i>cremoris</i>	Garde, et al. 1999
	<i>Lc. lactis</i> subsp. <i>lactis</i> and <i>Lc. lactis</i> subsp. <i>cremoris</i>	Nomura, et al. 2002
	Species and subspecies of <i>Lactococcus</i> genus	Pu, et al. 2002
	<i>Ln. mesenteroides</i> subsp. <i>mesenteroides</i>	Moschetti, et al. 2000
	<i>Ln. carnosum</i> , <i>Ln. citreum</i> and <i>Ln. mesenteroides</i> , <i>Ln. gelidum</i> and <i>Ln. lactis</i> .	Lee, et al. 2000
<i>Leuconostocs</i> (Ln.)	<i>Ln. mesenteroides</i> , <i>Ln. lactis</i> , <i>Ln. citreum</i> and <i>Weissella paramesenteroides</i>	Cibik, et al. 2000
<i>Propionibacterium</i>	Identification and differentiation of dairy propionibacteria and <i>P. acnes</i>	Rossi, et al. 1999

4 Concluding Remarks and Perspectives

In conclusion, the use of molecular tools in dairy microbiology has improved the knowledge on the succession of microbial strain and species during cheese manufacture and ripening. The culture-independent techniques can provide a rapid assessment of the microbial diversity while del culture-dependent molecular methods are of invaluable help in defining and monitoring microbial biotypes during important phases of cheese making. The sequence-based identification is expected to increase its impact and potential owing to the availability of high throughput sequencing platforms. The major perspective in the nearest future is the possibility to monitor not only the microorganisms, but also their activities during dairy fermentations. Owing to the introduction of real-time PCR systems coupled with the appropriate procedure of RNA extraction from food, it will hopefully be possible to understand which of the important activities are being carried out in a certain phase of the production and which are the environmental stresses affecting such activities. Therefore, the future target of food microbiology will be not only the wondering of “who is there,” but also

“who is doing what” with the final aim of improving technological processes and cheese quality and safety. Chemiometric and sensorial attributes should be associated to the evaluation of microbial diversity and activity in all the phases of interest to get a clear idea of the real association between microbiota, microbial metabolism and cheese quality. Therefore, only combined efforts of these approaches with proteomics, chemiometric measurements and sensorial evaluation can elucidate at an exploitable extent the complex and dynamic processes discussed here.

References

- Akopyanz, N., Bukanov, N.O., Westblom, T.U., Kresovich, S., Berg, D.E., 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res.* 20, 5137–5142.
- Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919–1925.
- Andrighetto, C., Borney, F., Barmaz, A., Stefanon, B., Lombardi, A., 2002. Genetic diversity of *Streptococcus thermophilus* strains isolated from Italian traditional cheeses. *Int. Dairy J.* 12, 141–144.
- Andrighetto, C., Psomas, E., Tzanetakis, N., Suzzi, G., Lombardi, A., 2000. Randomly amplified polymorphic DNA (RAPD) PCR for the identification of yeasts isolated from dairy products. *Lett. Appl. Microbiol.* 30, 5–9.
- Antonsson, M., Molin, G., Ardo, Y., 2003. *Lactobacillus* strains isolated from Danbo cheese adjunct cultures in a cheese model system. *Int. J. Food Microbiol.* 85, 159–169.
- Aquilanti, L., Dell’Aquila, L., Zannini, E., Zocchetti, A., Clementi F., 2006. Resident lactic acid bacteria in raw milk Canestrato Pugliese cheese. *Lett. Appl. Microbiol.* 43, 161–167.
- Aslam, Z., Im, W.T., Ten, L.N., Lee, Mj, Kim, K.H., Lee, S.T., 2006. *Lactobacillus siliginis* sp. nov., isolated from wheat sourdough in South Korea. *Int. J. Syst. Evol. Microbiol.* 56, 2209–2213.
- Aymerich, T., Martin, B., Garriga, M., Vidal-Carou, M.C., Bover-Cid, S., Hugas, M., 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J. Appl. Microbiol.* 100, 40–49.
- Baruzzi, F., Maturante, A., Morea, M., Cocconcetti, P.S., 2002. Microbial community dynamics during the Scamorza Altamura cheese natural fermentation. *J. Dairy Sci.* 85, 1390–1397.
- Baruzzi, F., Morea, M., Maturante, Cocconcetti, A., P.S., 2000. Changes in the *Lactobacillus* community during Ricotta forte cheese natural fermentation. *J. Appl. Microbiol.* 89, 807–814.
- Basaran, P., Basaran, N., Cakir, I., 2001. Molecular differentiation of *Lactococcus lactis* subspecies *lactis* and *cremoris* strains by Ribotyping and site specific-PCR. *Curr. Microbiol.* 42, 45–48.
- Beasley, S.S., Saris, P.E.J., 2004. Nisin-producing *Lactococcus lactis* strains isolated from human milk. *Appl. Environ. Microbiol.* 70, 5051–5053.
- Beresford, T.P., Fitzsimons, N.A., Brennan, N.L., Cogan, T.M., 2001. Recent advances in cheese microbiology. *Int. Dairy J.* 11, 259–274.
- Berthier, F., Beuvier, E., Dasen, A., Grappin, R., 2001. Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-specific primers. *Int. Dairy J.* 11, 293–305.
- Berthier, F., Ehrlich, S.D., 1998. Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. *FEMS Microbiol. Lett.* 161, 97–106.
- Berthier, F., Ehrlich, S.D., 1999. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. *Int. J. Syst. Bacteriol.* 49, 997–1007.

- Besancon, X., Smet, C., Chabalier, C., Rivemale, M., Reverbel, J.P., Ratomahenina, R., Galzy, P. 1992. Study of surface yeast flora of Roquefort cheese. *Int. Dairy J.* 17, 9–18.
- Blaiota, G., Pepe, O., Mauriello, G., Villani, F., Andolfi, R., Moschetti, G., 2002. 16S-23S rDNA intergenic spacer region polymorphism of *Lactococcus garvieae*, *Lactococcus raffinolactis* and *Lactococcus lactis* as revealed by PCR and nucleotide sequence analysis. *Syst. Appl. Microbiol.* 25, 520–527.
- Blaiota, G., Simeoli E., Andolfi R., Villani, F., Coppola, S., 2001. Monitoring lactic acid bacteria strains during “Cacioricotta” cheese production by restriction endonuclease analysis and pulsed-field gel electrophoresis. *J. Dairy Res.* 68, 139–144.
- Borgo, F., Ricci, G., Manachini, P.L., Fortina, M.G., 2007. Multilocus restriction typing: a tool for studying molecular diversity within *Lactobacillus helveticus* of dairy origin. *Int. Dairy J.* 17, 336–342.
- Bottari, B., Ercolini, D., Gatti, M., Neviani, E., 2006. Application of FISH technology for microbiological analysis: current state and prospects. *Appl. Microbiol. Biotechnol.* 73, 485–494.
- Bottazzi, V. 1993. Microbiologia e biotecnologia lattiero-casearia. Edagricole. Bologna, 157.
- Bouton, Y., Guyot, F., Beuvier, E., Tailliez, P., Grappin, R., 2002. Use of PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. *Int. J. Food Microbiol.* 76, 27–38.
- Brunner, S., Fleisch, F., Ruef, C., Altwegg, M., 2000. Automated ribotyping and pulsed-field gel electrophoresis reveal a cluster of group A streptococci in intravenous drug abusers. *Infection* 28, 314–317.
- Callon, C., Delbes, C., Duthoit, F., Montel, M.-C., 2006. Application of SSCP-PCR fingerprinting to profile yeast community in raw milk Salers cheese. *Syst. Appl. Microbiol.* 29, 172–180.
- Callon, C., Millet, L., Montel, M.-C., 2004. Diversity of lactic acid bacteria isolated from AOC Salers cheese. *J. Dairy Res.* 71, 231–244.
- Carvalho, M.G.S., Shewmaker, P.L., Steigerwalt, A.G., Morey, R.E., Sampson, A.J., Joyce, K., Barrett, T.J., Teixeira, L.M. and Facklam, R.R., 2006. *Enterococcus caccae* sp. nov., isolated from human stools. *Int. J. Syst. Evol. Microbiol.* 56, 1505–1508.
- Chagnaud, P., Machinis, K., Coutte, L.A., Marecat, A., Mercenier, A., 2001. Rapid PCR-based procedure to identify lactic acid bacteria: application to six common *Lactobacillus* species. *J. Microbiol. Meth.* 44, 139–148.
- Chambel, L., Chelo, I.M., Zé-zé, L., Pedro, L.G., Santos, M.A., Tenreiro, R., 2006. *Leuconostoc pseudoficulneum* sp. nov., isolated from a ripe fig. *Int. J. Syst. Evol. Microbiol.* 56, 1375–1381.
- Chan, R.K., Wortman, C.R., Smiley, B.K., Hendrick, C.A., 2003. Construction and use of a computerised DNA fingerprint database for lactic acid bacteria from silage. *J. Microbiol. Meth.* 55, 565–574.
- Chavagnat, F., Haueter, M., Jimeno, J., Casey, M. G., 2002. Comparison of partial *tuf* gene sequences for the identification of lactobacilli. *FEMS Microbiol. Lett.* 17, 177–183.
- Cheng, S., McCleskey, F.K., Gress, M.J., Petroziello, J.M., Liu, R., Namdari, H., Beninga, K., Salmen, A., Del Vecchio, V.G., 1997. A PCR assay for identification of *Enterococcus faecium*. *J. Clin. Microbiol.* 35, 1248–1250.
- Chun, S. R., Czajka J. W., Sakamoto M., Benno, Y., 2001. Characterization of the *Lactobacillus casei* group and the *Lactobacillus acidophilus* group by automated ribotyping. *Microbiol. Immunol.* 45, 271–275.
- Cibik, R., Lepage, E., Tailliez, P., 2000. Molecular diversity of *Leuconostoc mesenteroides* and *Leuconostoc citreum* isolated from traditional French cheeses as revealed by RAPD fingerprinting, 16S rDNA sequencing and 16S rDNA fragment amplification. *Syst. Appl. Microbiol.* 23, 267–278.
- Cocconcelli, P.S., Parisi, M.G., Senini, L., Bottazzi, V., 1997. Use of RAPD and 16S rDNA sequencing for the study of *Lactobacillus population* in natural whey culture. *Lett. Appl. Microbiol.* 25, 8–12.
- Cocconcelli, P.S., Porro, D., Galandini, S., Senini, L., 1995. Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. *Lett. Appl. Microbiol.* 21, 376–379.

- Cocolin, L., Innocente, N., Biasutti, M. and Comi, G., 2004. The late blowing in cheese: a new molecular approach based on PCR and DGGE to study the microbial ecology of the alteration process. *Int. J. Food Microbiol.* 90, 83–91.
- Coda, R., Brechany, E., De Angelis, M., De Candia, S., Di Cagno, R. and Gobbetti, M., 2006. Comparison of the compositional, microbiological, biochemical, and volatile profile characteristics of nine Italian ewe's milk cheeses. *J. Dairy Sci.* 89, 4126–4143.
- Coenye, T., Vandamme, P., 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol. Lett.* 228, 45–49.
- Coeurat, V., Dubernet, S., Bernardeau, M., Gueguen, M., Vernoux, J.P., 2003. Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait* 83, 269–306.
- Coppola, R., Nanni, M., Iorizzo, M., Sorrentino, A., Sorrentino, E., Chiavari, C., Grazia, L., 2000. Microbiological characteristics of Parmigiano Reggiano cheese during the cheesemaking and the first months of the ripening. *Lait*, 80, 479–490.
- Coppola, S., Blaiotta, G., Ercolini, D., Moschetti, G., 2001. Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. *J. Appl. Microbiol.* 90, 414–420.
- Coppola, S., Fusco, V., Andolfi, R., Aponte, M., Blaiotta, G., Ercolini, D., Moschetti, G., 2006. Evaluation of microbial diversity during the manufacture of Fior di Latte di Agerola, a traditional raw milk pasta-filata cheese of the Naples area. *J. Dairy Res.* 73, 264–272.
- Coppola, S., Parente, E., Dumontet, S. and La Peccerella, A., 1988. The microflora of natural whey cultures utilized as starter in the manufacture of Mozzarella cheese from water buffalo milk. *Lait* 68, 295–310.
- Corich, V., Mattiazzi, A., Soldati, E., Carraro, A., Giacomini A., 2005. Sau-PCR, a novel amplification technique for genetic fingerprinting of microorganisms. *Appl. Environ. Microbiol.* 71, 6401–6406.
- Corroler, D., Desmasures, N., Gueguen, M., 1999. Correlation between polymerase chain reaction analysis of the histidine biosynthesis operon, randomly amplified polymorphic DNA analysis and phenotypic characterization of dairy *Lactococcus isolates*. *Appl. Microbiol. Biotechnol.* 51, 91–99.
- Corroler, D., Mangin, I., Desmasures, N., Gueguen, M., 1998. An ecological study of lactococci isolated from raw milk in the Camembert cheese registered designation of origin area. *Appl. Environ. Microbiol.* 64, 4729–4735.
- Cosentino, S., Fadda, M.E., Deplano, M., Mulargia, A.F., Palmas, F., 2001. Yeasts associated with Sardinian ewe's dairy products. *Int. J. Food Microbiol.* 69, 53–58.
- Cosentino, S., Pisano, M.B., Corda, A., Fadda, M.E., Piras, C., 2004. Genotypic and technological characterization of enterococci isolated from artisanal Fiore Sardo cheese. *J. Dairy Res.* 71, 444–450.
- Cusick, S.M., O'Sullivan, D.J., 2000. Use of a single, triplicate arbitrarily primed-PCR procedure for molecular fingerprinting of lactic acid bacteria. *Appl. Environ. Microbiol.* 66, 2227–2231.
- Dawson, D., 2001. Molecular typing and identification of bacterial isolates. *New Food* 4, 63–65.
- De Angelis, M., Corsetti, A., Tosti, Rossi, J., Corbo M.R., Gobbetti, M., 2001. Characterization of non-starter lactic acid bacteria from Italian ewe cheeses based on phenotypic genotypic and cell wall protein analysis. *Appl. Environ. Microbiol.* 67, 2011–2020.
- Deasy, B.M., Rea, M.C., Fitzgerald, G.F., Cogan, T.M., Beresford, T.P., 2000. A rapid PCR based method to distinguish between *Lactococcus* and *Enterococcus*. *Syst. Appl. Microbiol.* 23, 510–522.
- De Las Rivas, B., Marcobal, A., Muñoz, R., 2006. Development of a multilocus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiol.* 152, 85–93.
- De Urraza, P. J., Gómez-Zavaglia, A., Lozano, M. E., Romanowski, V., De Antoni, G. L., 2000. DNA fingerprinting of thermophilic lactic acid bacteria using repetitive sequence-based polymerase chain reaction. *J. Dairy Res.* 67, 381–392.
- de Vin, F. Radstrom, P., Herman, L. De Vuyst, L., 2005. Molecular and biochemical analysis of the galactose phenotype of dairy *Streptococcus thermophilus* strains reveals four different fermentation profiles. *Appl. Environ. Microbiol.* 71, 3659–3667.
- Deiana, P. Personal communication. University of Sassari, Dipartimento Scienze Ambientali Sezione Microbiologia Agraria, viale Italia 39, Sassari, Italy.

- Delbes, C., Montel, M.-C., 2005. Design and application of a *Staphylococcus*-specific single strand conformation polymorphism-PCR analysis to monitor *Staphylococcus* populations diversity and dynamics during production of raw milk cheese. *Lett. Appl. Microbiol.* 41, 169–174.
- Delgado, S., Mayo, B., 2004. Phenotypic and genetic diversity of *Lactococcus lactis* and *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse cheeses. *Int. J. Food Microbiol.* 90, 309–319.
- Dellaglio, F., Vancanneyt, M., Endo, A., Vandamme, P., Felis, G. E., Castioni, A., Fujimoto, J., Watanabe, K., Okada S., 2006. *Lactobacillus durianis* Leisner *et al.* 2002 is a later heterotypic synonym of *Lactobacillus vaccinostrercus* Kozaki and Okada 1983. *Int. J. Syst. Evol. Microbiol.* 56, 1721–1724.
- Dimitrov, Z., Michaylova, M., Mincova, S., 2005. Characterization of *Lactobacillus helveticus* strains isolated from Bulgarian yoghurt, cheese, plants and human faecal samples by sodium dodecylsulfate polyacrylamide gel electrophoresis of cell-wall proteins, ribotyping and pulsed field gel fingerprinting. *Int. Dairy J.* 15, 998–1005.
- Dobson, C.M., Chaban, B., Deneer, H., Ziola, B.R., 2004. *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus zeae* isolates identified by sequence signature and immunoblot phenotype. *Can. J. Microbiol.* 50, 482–488.
- Domig, K.J., Mayer, H.K., Kneifel, W., 2003. Methods used for the isolation, enumeration, characterisation and identification of *Enterococcus* spp. 2. Pheno- and genotypic criteria. *Inter. J. Food Microbiol.* 88, 165–188.
- Drake, M., Small, C.L., Spence, K.D., Swanson, B.G., 1996a. Differentiation of *Lactobacillus helveticus* strains using molecular typing methods. *Food Res. Int.* 29, 451–455.
- Drake, M., Small, C.L., Spence, K.D., Swanson, B.G., 1996b. Rapid detection and identification of *Lactobacillus* spp. in dairy products using repetitive sequence based polymerase chain reaction. *J. Food Prot.* 59, 1031–1036.
- Dupré, I., Zanetti, S., Schito, A.M., Fadda, G., Sechi, L.A., 2003. Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J. Med. Microbiol.* 52, 491–498.
- Duthoit, F., Callon, C., Tessier, L., Montel, M.-C., 2005a. Relationships between sensorial characteristics and microbial dynamics in “Registered Designation Origin” Salers cheese. *Int. J. Food Microbiol.* 103, 259–270.
- Duthoit, F., Godon, J.-J., Montel, M.-C., 2003. Bacterial community dynamics during production of Registered Designation Origin Salers cheese as evaluated by 16S rRNA gene by single-strand conformation polymorphism analysis. *Appl. Environ. Microbiol.* 69, 3840–3848.
- Duthoit, F., Tessier, L., Montel, M.-C., 2005b. Diversity dynamics and activity of bacterial populations in “Registered Designation Origin” Salers cheese by single-strand conformation polymorphism analysis of 16S rRNA genes. *J. Appl. Microbiol.* 98, 1198–1208.
- Dutka-Malen, S., Evers, S., Courvalin, P., 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J. Clin. Microbiol.* 33, 24–27.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Meth.* 56, 297–314.
- Ercolini, D., Blaiotta, G., Moschetti, G., Coppola, S., 2002. Molecular typing of cheeses on the basis of their microflora as detected by PCR-DGGE analysis. *Ann. Microbiol.* 52, 81–87.
- Ercolini, D., Fusco, V., Blaiotta, G., Coppola, S., 2005. Sequence heterogeneity in the *lacSZ* operon of *Streptococcus thermophilus* and its use in PCR systems for strain differentiation. *Res. Microbiol.* 156, 161–172.
- Ercolini, D., Hill, P.J., Dodd, C.E.R., 2003a. Bacterial community structure and location in Stilton cheese. *Appl. Environ. Microbiol.* 69, 3540–3548.
- Ercolini, D., Hill, P.J., Dodd, C.E.R., 2003b. Development of a fluorescence in situ hybridisation method for cheese using a 16S rRNA probe. *J. Microbiol. Meth.* 52, 267–271.
- Ercolini, D., Mauriello, G., Blaiotta, G., Moschetti, G., Coppola, S., 2004. PCR-DGGE fingerprints of microbial succession during a manufacture of traditional water buffalo Mozzarella cheese. *J. Appl. Microbiol.* 96, 263–270.

- Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. *Syst. Appl. Microbiol.* 24, 610–617.
- Erlandson, K., Batt, C.A., 1997. Strain specific differentiation of lactococci in mixed starter culture populations using randomly amplified polymorphic DNA-derived probes. *Appl. Environ. Microbiol.* 63, 2702–2707.
- Farrelly, M.E., Rainey, F.A., Stackebrandt, E., 1995. Effect of genome size and *rrn* gene copy number in PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microbiol.* 61, 2798–2801.
- Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., Torriani, S., 2003. Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int. J. Food Microbiol.* 82, 59–70.
- Felis, G.E., Dellaglio, F., 2005. Taxonomy of lactobacilli and bifidobacteria. In: Tannock G.W. Editor, *Probiotics and prebiotics: scientific aspects*. Caster Academic Press, Norfolk, UK.
- Felis, G.E., Vancanneyt, M., Snauwaert, C., Swings, J., Torriani, S., Castioni, A., Dellaglio, F., 2006. Reclassification of *Lactobacillus thermotolerans* Niamsup *et al.* 2003 as a later synonym of *Lactobacillus ingluviei* Baele *et al.* 2003. *Int. J. Syst. Evol. Microbiol.* 56, 793–795.
- Fernandez, M., Linares, D.M., Alvarez, M.A., 2004. Sequencing of the tyrosine decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. *J. Food Prot.* 67, 2521–2529.
- Fitzsimons, N.A., Cogan, T.M., Condon, S., Beresford, T., 1999. Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature cheddar cheese. *Appl. Environ. Microbiol.* 65:3218–3426.
- Flint, J.F., Angert, E.R., 2005. Development of a strain-specific assay for detection of viable *Lactobacillus* sp. HOFG1 after application to cattle feed. *J. Microbiol. Meth.* 61, 235–243.
- Fortina, M.G., Ricci, G., Mora, D., Parini, C., Manachini, P.L., 2001. Specific identification of *Lactobacillus helveticus* by PCR with *pepC*, *pepN* and *htrA* targeted primers. *FEMS Microbiol. Lett.* 198, 85–89.
- Fortina, M.G., Ricci, G., Acquati A., Zeppa G., Gandini, A., Manachini, P.L., 2003. Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese. *Food Microbiol.* 20, 397–404.
- Fortina, M.G., Ricci, G., Mora, D., Manachini, P.L., 2004. Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov.. *Int. J. Syst. Evol. Microbiol.* 54, 1717–1721.
- Fox, P.F., McSweeney, P.L.H., 2004. Cheese: An overview. In: Fox, P.H., McSweeney, P.L.H., Cogan, T. and Guinee, T. (Eds.), *Cheese: Chemistry, Physics & Microbiology*. Elsevier Academic Press, San Diego. 1–18.
- Franz, C.M.A.P., Vancanneyt, M., Vandemeulebroecke, K., De Wachter, M., Cleenwerck, I., Hoste, B., Schillinger, U., Holzapfel, W.H., Swings, J., 2006. *Pediococcus stilesii* sp. nov., isolated from maize grains. *Int. J. Syst. Evol. Microbiol.* 56, 329–333.
- Friedrich, U., Lenke, J., 2006. Improved enumeration of lactic acid bacteria in mesophilic dairy starter cultures by using multiplex quantitative real-time PCR and flow cytometry-fluorescence in situ hybridization. *Appl. Environ. Microbiol.*, 72, 4163–4171.
- Gancheva, A., Pot, B., Vanhonacker, K., Hoste, B., Kersters, K., 1999. A polyphasic approach towards identification of strains belonging to *Lactobacillus acidophilus* and related species. *Syst. Appl. Microbiol.* 22, 573–585.
- Garde, S., Babin, M., Gaya, P., Nunez, M., Medina, M., 1999. PCR amplification of the gene *acmA* differentiates *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. *Appl. Environ. Microbiol.* 65, 5151–5153.
- Gatti, M., Rossetti, L., Fornasari, M.E., Lazzi, C., Giraffa, G., Neviani, E., 2005. Heterogeneity of putative surface layer proteins in *Lactobacillus helveticus*. *Appl. Environ. Microbiol.* 71, 7582–7588.
- Gatti, M., Trivisano, C., Fabrizi, E., Neviani, E., Gradini, F., 2004. Biodiversity among *Lactobacillus helveticus* strains isolated from different Natural Whey Starter Cultures as revealed by classification trees. *Appl. Environ. Microbiol.* 70, 182–190.

- Gelsomino, R., Vancanneyt, M., Cogan, T. M., Condon, S., Swings, J., 2002. Source of enterococci in a farmhouse raw-milk cheese. *Appl. Environ. Microbiol.* 68, 3560–3565.
- Gelsomino, R., Vancanneyt, M., Condon, S., Swings, J., Cogan, T. M., 2001. Enterococcal diversity in the environment of an Irish Cheddar-type cheesemaking factory. *Int. J. Food Microbiol.* 71, 177–188.
- Giraffa, G., Andrighetto, C., Antonello, C., Gatti, M., Lazzi, C., Marcazzan, G., Lombardi, A., Neviani, E., 2004. Genotypic and phenotypic diversity of *Lactobacillus delbrueckii* subsp. *lactis* strains of dairy origin. *Int. J. Food Microbiol.* 91, 29–139.
- Giraffa, G., Paris, A., Valcavi, L., Gatti, M., Neviani, E., 2001. Genotypic and phenotypic heterogeneity of *Streptococcus thermophilus* strains isolated from dairy products. *J. Appl. Microbiol.* 91, 937–943.
- Glazunova, O.O., Raoult, D., Roux, V., 2006. *Streptococcus massiliensis* sp. nov., isolated from a patient blood culture. *Int. J. Syst. Evol. Microbiol.* 56, 1127–1131.
- Gobbetti, M., Morea, M., Baruzzi, F., Corbo, M.R., Matarante, A., Considine, T., Di Cagno, R., Guinee, T., Fox, P.F., 2002. Microbiological, compositional, biochemical and textural characterisation of Caciocavallo Pugliese cheese during ripening. *Int. Dairy J.* 12, 511–523.
- Goh, S.H., Facklam, R.R., Chang, M., Hill, J.E., Tyrrell, G.J., Burns, C.M., Chan, D., He, C., Rahim, T., Shaw, C., Hemmingsen, S.M., 2000. Identification of *Enterococcus* species and phenotypically similar *Lactococcus* and *Vagococcus* species by reverse checkerboard hybridisation to chaperonin 60 gene sequences. *J. Clin. Microbiol.* 38, 3953–3959.
- Grif, K., Dierich, M.P., Much, P., Hofer, E., Allerberger, F., 2003. Identifying and subtyping species of dangerous pathogens by automated ribotyping. *Diagn. Microbiol. Infect. Dis.* 47, 313–320.
- Grimont, F., Grimont, P.A.D., 1986. Ribosomal ribonucleic acid gene restriction as potential taxonomic tools. *Annales de l'Institut Pasteur/ Microbiologie* 137 B, 165–175.
- Guarneri, T., Rossetti, L., Giraffa, G., 2001. Rapid identification of *Lactobacillus brevis* using the polymerase chain reaction. *Lett. Appl. Microbiol.* 33, 377–381.
- Huey, B., Hall, J., 1989. Hypervariable DNA fingerprinting in *E. coli*. Minisatellite probe from bacteriophage M13. *J. Bacteriol.* 171, 2528–2532.
- Itoh, Y., Kawamura, Y., Kasai, H., Shah, M. M., Nhung, P. H., Yamada, M., Sun, X., Koyana, T., Hayashi, M., Ohkusu, K., Ezaki, T., 2006. *dnaJ* and *gyrB* gene sequence relationship among species and strains of genus *Streptococcus*. *Syst. Appl. Microbiol.* 29, 368–374.
- Jayarao, B.M., Dore, J.J.E., Oliver, S.P., 1992. Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. *J. Clin. Microbiol.* 30, 2235–2240.
- Jenkins, J.K., Harper W.J., Courtney, P.D., 2002. Genetic diversity in Swiss cheese starter cultures assessed by pulsed field gel electrophoresis and arbitrarily primed PCR. *Lett. Appl. Microbiol.* 35, 423–427.
- Jensen, M.A., Webster, J.A., Strauss, N., 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* 59, 945–952.
- Johansson, M.L., Quednau, M., Molin, G., Ahrne, S., 1995. Randomly Amplified Polymorphic DNA (RAPD) for typing of *Lactobacillus plantarum* strains. *Lett. Appl. Microbiol.* 21, 155–159.
- Johnson, M.E. 1998. Cheese products. In: Marth, E.H. and Steel, J.L. (Eds.), *Applied dairy microbiology*. Marcel Dekker, Inc., New York. 213–249.
- Johnson, M.E. and Steele, J.L. 1997. Fermented dairy products. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J. (Eds.), *Food Microbiology*, ASM Press, Washington D.C., 581–594.
- Jurkovic, D., Krizkova, L., Dusinsky, R., Belicova, A., Sojka, M., Krajcovic, J., Ebringer, L., 2006. Identification and characterization of enterococci from bryndza cheese. *Lett. Appl. Microbiol.* 42, 553–559.
- Ke, D., Picard, F.J., Martineau, F., Menard, C., Roy, P.H., Ouellette, M., Bergeron, M.G., 1999. Development of a PCR assay for rapid detection of enterococci. *J. Clin. Microbiol.* 37, 3497–3503.
- Khan, S.A., Nawaz, M.S., Khan, A.A., Hopper, S.L., Jones, R. A., Cerniglia C. E., 2005. Molecular characterization of multidrug-resistant *Enterococcus* spp. from poultry and dairy farms: detection of virulence and vancomycin resistance gene markers by PCR. *Mol. Cell. Probes* 19, 27–34.

- Kitahara, M., Sakata, S., Benno, Y., 2005. Biodiversity of *Lactobacillus sanfranciscensis* strains isolated from five sourdoughs. *Lett. Appl. Microbiol.* 40, 353–357.
- Klijn, N., Nieuwendorf, F.F.J., Hoolwerf, J.D., van der Waals, C.B., Weerkamp, A.H., 1995a. Identification of *Clostridium tyrobutyricum* as the causative agent of late blowing in cheese by species-specific PCR amplification. *Appl. Environ. Microbiol.*, 61, 2919–2924.
- Klijn, N., Weerkamp, A.H., de Vos, W.M., 1995. Detection and characterization of lactose-utilizing *Lactococcus* subsp. in natural ecosystems. *Appl. Environ. Microbiol.* 61, 788–792.
- Knijff, E., Dellaglio, F., Lombardi, A., Andrighetto, C., Torriani, S., 2001. Rapid identification of *Enterococcus durans* and *Enterococcus hirae* by PCR with primers targeted to the *ddl* genes. *J. Microbiol. Meth.* 47, 35–40.
- Konstantinov, S.R., Poznanski, E., Fuentes, S., Akkermans, A.D.L., Smidt, H., De Vos, W.M., 2006. *Lactobacillus sobrius* sp. nov., abundant in the intestine of weaning piglets. *Int. J. Sys. Evol. Microbiol.* 56, 29–32.
- Kwon, H.S., Yang, E.H., Yeon, S.W., Kang, B.H., Kim, T.Y., 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. *FEMS Microbiol. Lett.* 239, 267–75.
- Lafarge, V., Ogier, J.-C., Girard, V., Maladen, V., Leveau J.-Y., Gruss, A., Delacroix-Buchet, A., 2004. Raw cow milk bacterial population shifts attributable to refrigeration. *Appl. Environ. Microbiol.* 70, 5644–5650.
- Lazzi, C., Rossetti, L., Zago, M., Neviani E., Giraffa, G., 2004. Evaluation of bacterial communities belonging to natural whey starters for Grana Padano cheese by length heterogeneity-PCR. *J. Appl. Microbiol.* 96, 481–490.
- Lee, H.J., Park, S.Y., Kim, J., 2000. Multiplex PCR-based detection and identification of *Leuconostoc* species. *FEMS Microbiol. Lett.* 193, 243–247.
- Leisner, J.J., Pot, B., Christensen, H., Rusul, G., Olsen, J.E., Wee, B.W., Muhamad, K., Ghazali, H.M., 1999. Identification of lactic acid bacteria from chili bo, a Malaysian food ingredient. *Appl. Environ. Microbiol.* 65, 599–605.
- Lick, S., Keller, M., Bockelmann, W., Jochem Heller, K., 1996. Rapid identification of *Streptococcus thermophilus* by primerspecific PCR amplification based on its *lacZ* gene. *Syst. Appl. Microbiol.* 19, 74–77.
- Limosowtin, G.K.Y., Powell, I.B., Parente, E., 1996. Types of starters. In: Cogan, T.M., Accolas, J.-P. (Eds.). *Dairy starter cultures*. Wiley-VCH, New York. 101–130.
- Lombardi, A., Gatti, M., Rizzotti, L., Torriani, S., Andrighetto, C., Giraffa, G., 2004. Characterization of *Streptococcus macedonicus* strains isolated from artisanal Italian raw milk cheeses. *Int. Dairy J.* 14, 967–976.
- Mangin, I., Corroler, D., Reinhardt, A., Gueguen, M., 1999. Genetic diversity among dairy lactococcal strains investigated by polymerase chain reaction with three arbitrary primers. *J. Appl. Microbiol.* 86, 514–520.
- Mannu, L., Comunian, R., Scintu, M.F., 2000a. Mesophilic lactobacilli in Fiore Sardo cheese: PCR-identification and evolution during cheese ripening. *Int. Dairy J.* 10, 383–389.
- Mannu, L., Paba, A., 2002. Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewes' milk cheese. *J. Appl. Microbiol.*, 92, 55–62.
- Mannu, L., Paba, A., Pes, M., Scintu, M.F., 2000b. Genotypic and phenotypic heterogeneity among lactococci isolated from traditional Pecorino Sardo cheese. *J. Appl. Microbiol.* 89, 191–197.
- Marcobal, A., de Las Rivas, B., Moreno-Arribas, M.V., Munoz, R., 2005. Multiplex PCR method for the simultaneous detection of histamine-, tyramine-, and putrescine-producing lactic acid bacteria in foods. *J. Food Prot.* 68, 874–878.
- Massi, M., Vitali, B., Federici, F., Matteuzzi, D., Brigidi, P., 2004. Identification method based on PCR combined with automated ribotyping for tracking probiotic *Lactobacillus* strains colonizing the human gut and vagina. *J. Appl. Microbiol.* 96, 777–786.
- Matte-Tailliez, O., Lepage, E., Tenenhaus, M., Tailliez, P., 2002. Use of Predictive Modeling for *Propionibacterium* Strain Classification. *Syst. Appl. Microbiol.* 25, 386–395.
- Mauriello, G., Moio, L., Genovese, A., Ercolini, D., 2003. Relationship between flavouring capabilities, bacterial composition and geographical origin of Natural Whey Cultures (NWCs) used for water-buffalo Mozzarella cheese manufacture. *J. Dairy Sci.* 86, 486–497.

- Mauriello, G., Moio, L., Moschetti, G., Piombino, P., Addeo, F., Coppola, S., 2001. Characterization of lactic acid bacteria strains on the basis of neutral volatile compounds produced in whey. *J. Appl. Microbiol.* 90, 928–942.
- McSweeney, P.L.H., Ottogalli, G. and Fox, P.F. 2004. Diversity of cheese varieties: An overview. In: Fox, P.H., McSweeney, P.L.H., Cogan, T. and Guinee, T. (Eds.), *Cheese: Chemistry, Physics & Microbiology*. Elsevier Academic Press, San Diego. Vol. 2, 1–22.
- Mora, D., Fortina, M.G., Parini, C., Ricci, G., Gatti, M., Giraffa, G., Manachini, P. L., 2002a. Genetic diversity and technological properties of *Streptococcus thermophilus* strains isolated from dairy products. *J. Appl. Microbiol.* 93, 278–287.
- Mora, D., Parini, C., Fortina, M.G., Manachini, P.L., 2002b. Multilocus hybridization typing in *Pediococcus acidilactici* strains. *Curr. Microbiol.* 44, 77–80.
- Mora, D., Ricci, G., Guglielmetti, S., Daffonchio, D., Fortina, M. G., 2003. 16S–23S rRNA intergenic spacer region sequence variation in *Streptococcus thermophilus* and related dairy streptococci and development of a multiplex ITS-SSCP analysis for their identification. *Microbiol.* 149, 807–813.
- Morandi, S., Brasca, M., Andrighetto, C., Lombardi, A., Lodi, R., 2006. Technological and molecular characterisation of enterococci isolated from north–west Italian dairy products. *Int. Dairy J.* 16, 867–875.
- Morea, M., Baruzzi, F., Cappa, F., Coconcelli, P.S. 1998. Molecular characterization of the *Lactobacillus* community in traditional processing of Mozzarella cheese. *Int. J. Food Microbiol.* 43, 53–60.
- Morea, M., Baruzzi, F., Coconcelli, P.S., 1999. Molecular and physiological characterization of dominant bacterial populations in traditional Mozzarella cheese processing. *J. Appl. Microbiol.* 87, 574–582.
- Moreira, J.L., Mota, R.M., Horta, M.F., Teixeira, S.M., Neumann, E., Nicoli, J.R., Nunes, A.C., 2005. Identification to the species level of *Lactobacillus* isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. *BMC Microbiol.* 5, 15–20.
- Moschetti, G., Blaiotta, G., Aponte, M., Catzeddu, P., Villani, F., Deiana, P., Coppola, S., 1998. Random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA spacer polymorphism: powerful methods to differentiate *Streptococcus thermophilus* strains. *J. Appl. Microbiol.* 85, 25–36.
- Moschetti, G., Blaiotta, G., Aponte, M., Mauriello, G., Villani, F., Coppola, S., 1997. Genotyping of *Lactobacillus delbrueckii* subsp. *bulgaricus* and determination of number and forms of *rrn* operons in *Lactobacillus delbrueckii* and its subspecies. *Res. Microbiol.* 148, 501–510.
- Moschetti, G., Blaiotta, G., Villani, F., Coppola, S., 2000. Specific detection of *Leuconostoc mesenteroides* subsp. *mesenteroides* with DNA primers identified by random amplified polymorphic DNA (RAPD) analysis. *Appl. Environ. Microbiol.* 66, 422–424.
- Moschetti, G., Blaiotta, G., Villani, F., Coppola, S., 2001. Nisin-producing organisms during traditional ‘Fior di latte’ cheese-making monitored by multiplex-PCR and PFGE analyses. *Int. J. Food Microbiol.* 63, 109–116.
- Mounier, J., Gelsomino, R., Goerges, S., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., Scherer, S., Swings, J., Fitzgerald, G.F., Cogan, T.M., 2005. Surface microflora of four smear-ripened cheeses. *Appl. Environ. Microbiol.* 71, 6489–6500.
- Mounier, J., Goerges, S., Gelsomino, R., Vancanneyt, M., Vandemeulebroecke, K., Hoste, B., Brennan, N.M., Scherer, S., Swings, J., Fitzgerald, G.F., Cogan, T.M., 2006. Sources of the adventitious microflora of a smear-ripened cheese. *J. Appl. Microbiol.* 101, 668–681.
- Mucchetti, G. and Neviani, E., 2006. *Microbiologia e tecnologia lattiero-casearia. Qualità e sicurezza. Tecniche Nuove*, Milano.
- Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Thompson, C.C., Vancanneyt, M., Swings, J., 2005a. Phylogeny and Identification of *Enterococci* by *atpA* Gene Sequence Analysis. *J. Clin. Microbiol.* 43, 2224–2230.
- Naser, S.M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J., 2005b. Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiol.* 151, 2141–2150.

- Naser, S.M., Hagen, K.E., Vancanneyt, M., Cleenwerck, I., Swings, J., Tompkins, T.A., 2006a. *Lactobacillus suntoryeus* Cachat and Priest 2005 is a later synonym of *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey *et al.* 1925 (Approved Lists 1980). *Int. J. Syst. Evol. Microbiol.* 56, 355–360.
- Naser, S.M., Vancanneyt, M., Hoste, B., Snauwaert, C., Swings, J., 2006b. *Lactobacillus cypriacae* Lawson *et al.* 2001 is a later heterotypic synonym of *Lactobacillus acidipiscis* Tanasupawat *et al.* 2000. *Int. J. Syst. Evol. Microbiol.* 56, 1681–1683.
- Naser, S.M., Vancanneyt, M., Hoste, B., Snauwaert, C., Vandemeulebroecke, K., Swings, J., 2006c. Reclassification of *Enterococcus flavescens* Pompei *et al.* 1992 as a later synonym of *Enterococcus casseliflavus* (ex Vaughan *et al.* 1979) Collins *et al.* 1984 and *Enterococcus saccharominimus* Vancanneyt *et al.* 2004 as a later synonym of *Enterococcus italicus* Fortina *et al.* 2004. *Int. J. Syst. Evol. Microbiol.* 56, 413–416.
- Nigatu, A., Ahrné, S., Gashe, B.A., Molin, G., 1998. Randomly amplified polymorphic DNA (RAPD) for discrimination of *Pediococcus pentosaceus* and *Ped. acidilactici* and rapid grouping of *Pediococcus* isolates. *Lett. Appl. Microbiol.* 26, 412–416.
- Nomura, M., Kobayashi, M., Narita, T., Kimoto-Nira, H., Okamoto, T., 2006. Phenotypic and molecular characterization of *Lactococcus lactis* from milk and plants. *J. Appl. Microbiol.* 101, 396–405.
- Nomura, M., Kobayashi, M., Okamoto, T., 2002. Rapid PCR-based method which can determine both phenotype and genotype of *Lactococcus lactis* subspecies. *Appl. Environ. Microbiol.* 68, 2209–2213.
- Nooigedagt, A.J., Hartog, B.J., 1988. A survey of the microbiological quality of Brie and Camembert cheese. *Neth. Milk Dairy J.* 42, 57–72.
- O'Sullivan, T.F., Fitzgerald, G.F., 1998. Comparison of *Streptococcus thermophilus* strains by pulse field gel electrophoresis of genomic DNA. *FEMS Microbiol. Lett.* 168, 213–219.
- O'Sullivan, D.J., 1999. Methods for analysis of the intestinal microflora. In: Tannock, G.W. (Ed.), *Probiotics: A critical review*. Horizon Scientific Press, Wymondham, 23–44.
- O'Sullivan, L., O'Connor, E.B., Ross, R.P., Hill, C., 2006. Evaluation of live-culture-producing lacticin 3147 as a treatment for the control of *Listeria monocytogenes* on the surface of smear-ripened cheese. *J. Appl. Microbiol.* 100, 135–143.
- Oberman, H., Libudzisz, Z., 1998. Fermented milks. In: Wood, B.J.B. (Ed.), *Microbiology of fermented foods*. Blackie Academic & Professional, London, 1, 308–350.
- Obodai, M., Dodd, C.E.R., 2006. Characterization of dominant microbiota of a Ghanaian fermented milk product, *nyarmie*, by culture- and non culture-based methods. *J. Appl. Microbiol.* 100, 1355–1363.
- Ogier, J.-C., Son, O., Gruss, A., Tailliez, P., Delacroix-Buchet, A., 2002. Identification of bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 68, 3691–3701.
- Ogier, J.-C., Lafarge, V., Girard, V., Rault, A., Maladen, V., Gruss, A., Leveau J.-Y., Delacroix-Buchet, A., 2004. Molecular fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70, 5628–5643.
- Osawa, R., Fujisawa, T., Pukall, R., 2006. *Lactobacillus apodemi* sp. nov., a tannase-producing species isolated from wild mouse faeces. *Int. J. Syst. Evol. Microbiol.* 56, 1693–1696.
- Ottogalli, G., 2001. *Atlante dei formaggi*. Hoepli, Milan. 31–39.
- Pacini, F., Cariolato, D., Andrighetto, C., Lombardi, A., 2006. Occurrence of *Streptococcus macedonicus* in Italian cheeses. *FEMS Microbiol. Lett.* 261, 69–73.
- Paffetti, D., Barberio, C., Casalone, E., Cavalieri, D., Fani, R., Fia, G., Mori, E., Polsinelli, M., 1995. DNA fingerprinting by random amplified polymorphic DNA and restriction fragment length polymorphism is useful for yeast typing. *Res. Microbiol.* 146, 587–594.
- Papadelli, M., Manolopoulou, E., Kalantzopoulos, G., Tsakalidou, E., 2003. Rapid detection and identification of *Streptococcus macedonicus* by species-specific PCR and DNA hybridization. *Int. J. Food Microbiol.* 81, 231–239.
- Parayre, S., Falentin, H., Madec, M.-N., Sivieri, K., Le Dizes, A.-S., Shoier, D., Lortal, S., 2007. Easy DNA extraction method and optimization of PCR-Temporal temperature gradient gel

- electrophoresis to identify the predominant high and low GC-content bacteria from dairy products. *J. Microbiol. Meth.* 69, 431–441.
- Pérez, G., Cardell, E., Zárate, V., 2000. Protein fingerprinting as a complementary analysis to classical phenotyping for the identification of lactic acid bacteria from Tenerife cheese. *Lait* 80, 589–600.
- Piraino, P., Ricciardi, A., Salzano, G., Zotta, T., Parente, E., 2006. Use of unsupervised and supervised artificial neural networks for the identification of lactic acid bacteria on the basis of SDS-PAGE patterns of whole cell proteins. *J. Microbiol. Meth.* 66, 336–346.
- Piraino, P., Zotta, T., Ricciardi, A., Parente, E., 2005. Discrimination of commercial Caciocavallo cheeses on the basis of the diversity of lactic microflora and primary proteolysis. *Int. Dairy J.* 15, 1138–1149.
- Pot, B., Vandamme, P., Kersters, K., 1994. Analysis of electrophoretic whole-organism protein fingerprints. In: Goodfellow, M., O'Donnell, A.G. (Eds.), *Chemical Methods in Prokaryotic Systematics*. John Wiley and Sons, New York.
- Poyart, C., Quesnes, G., Trieu-Cuot, P., 2000. Sequencing the gene encoding manganese-dependent superoxide dismutase for rapid species identification of enterococci. *J. Clin. Microbiol.* 38, 415–418.
- Poznanski, E., Cavazza, A., Cappa, F., Cocconcelli, P.S., 2004. Indigenous raw milk microbiota influences the bacterial development in traditional cheese from an alpine natural park. *Int. J. Food Microbiol.* 92, 141–151.
- Psoni, L., Kotzamanides, C., Andrighetto, C., Lombardi, A., Tzanetakis, N., Litopoulou-Tzanetaki, E., 2006. Genotypic and phenotypic heterogeneity in *Enterococcus* from Batzos, a raw goat milk cheese. *Int. J. Food Microbiol.* 109, 109–120.
- Pu, Z.Y., Dobos, m., Limsowtin, G.K.Y., Powell, I.B., 2002. Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the gram-positive bacterial genus *Lactococcus*. *J. Appl. Microbiol.* 93, 353–361.
- Quere, F., Deschamps, A., Urdaci, M.C., 1997. DNA probe and PCR-specific reaction for *Lactobacillus plantarum*. *J. Appl. Microbiol.* 82, 783–790.
- Randazzo, C.L., Torriani, S., Akkermans, A.D.L., de Vos, W.M., Vaughan, E.E., 2002. Diversity, dynamics and activity of bacterial communities during production of an artisanal sicilian cheese as evaluated by 16S rRNA analysis. *Appl. Environ. Microbiol.* 68, 1882–1892.
- Randazzo, C.L., Vaughan, E.E., Caggia, C., 2006. Artisanal and experimental Pecorino Siciliano cheese: microbial dynamics during manufacture assessed by culturing and PCR-DGGE analyses. *Int. J. Food Microbiol.* 109, 1–8.
- Rantsiou, K., Comi, G., Cocolin, L., 2004. The *rpoB* gene as a target for PCR-DGGE analysis to follow lactic acid bacterial population dynamics during food fermentation. *Food Microbiol.* 21, 481–487.
- Reinheimer, J. A., Quiberoni, A., Taillez, P., Binetti, A.G., Suarez, V.B., 1996. The lactic acid microflora of natural whey starters used in Argentina for hard cheese production. *Int. Dairy J.* 6, 869–879.
- Reysenbach, A.L., Giver, L.J., Wickham, G.S., Pace, N.R., 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58, 3417–3418.
- Rodas, A.M., Chenoll, E., Macián, M.C., Ferrer, S., Pardo, I. Aznar, R., 2006. *Lactobacillus vini* sp. nov., a wine lactic acid bacterium homofermentative for pentoses. *Int. J. Syst. Evol. Microbiol.* 56, 513–517.
- Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. *J. Microbiol. Meth.* 63, 135–144.
- Rossi, F., Dellaglio, F., Torriani, S., 2006. Evaluation of *recA* gene as a phylogenetic marker in the classification of dairy propionibacteria. *Syst. Appl. Microbiol.* 29, 463–469.
- Rossi, F., Torriani, S., Dellaglio, F., 1998. Identification and clustering of dairy propionibacteria by RAPD-PCR and CGE-REA methods. *J. Appl. Microbiol.* 85, 956–964.
- Rossi, F., Torriani, S., Dellaglio, F., 1999. Genus- and species-specific PCR-based detection of dairy propionibacteria in environmental samples by using primers targeted to the genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 65, 4241–4244.

- Saleh-Lakha, S., Miller, M., Campbell, R.G., Schneider, K., Elahimanesh, P., Hart, M.M., Trevors, J.T., 2005. Microbial gene expression in soil: methods, applications and challenges. *J. Microbiol. Meth.* 63, 1–19.
- Sanchez, I., Sesena, S., Palop, L.I., 2004. Polyphasic study of the genetic diversity of lactobacilli associated with “Al magro” eggplants spontaneous fermentation, based on combined numerical analysis of randomly amplified polymorphic DNA and pulsed-field gel electrophoresis patterns. *J. Appl. Microbiol.* 97, 446–458.
- Sanchez, I., Sesena, S., Poveda, J. M., Cabezas, L., Palop, L.I., 2005. Phenotypic and genotypic characterization of lactobacilli isolated from Spanish goat cheeses. *Int. J. Food Microbiol.* 102, 355–362.
- Sánchez, I., Sesena, S., Poveda, J.M., Cabezas, L., Palop, L., 2006. Genetic diversity, dynamics, and activity of *Lactobacillus* community involved in traditional processing of artisanal Manchego cheese. *Int. J. Food Microbiol.* 107, 265–273.
- Silva, J., Carvalho, A.S., Duarte, G., Lopes, Z., Domingues, P., Teixeira, P., Gibbs, P.A., 2003. Characterisation of enterococci strains isolated from an artisanal Portuguese cheese. *Milchwissenschaft* 58, 389–392.
- Simpson, P. J., Stanton, C., Fitzgerald, G.F., Ross, R. P., 2002. Genomic diversity within the genus *Pediococcus* as Revealed by randomly amplified polymorphic DNA PCR and pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* 68, 765–771.
- Spiegelman, D., Wissell, G., Greer, C.W., 2005. A survey of the methods for the characterization of microbial consortia and communities. *Can. J. Microbiol.* 51, 355–386.
- Succi, M., Tremonte P., Reale, A., Sorrentino, E., Grazia, L., Pacifico, S., Coppola, R., 2005. Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese. *FEMS Microbiol. Lett.* 244, 129–137.
- Suzuki, M.T., Giovannoni, S.J., 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* 62, 625–630.
- Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M.E., Andrighetto, Lanorte, C., M.T., 2000. A survey of the enterococci isolated from an artisanal Italian goat's cheese (semicotto caprino). *J. Appl. Microbiol.* 89, 267–274.
- Svec, P., Drab, B., Sedláček, I., 2005. Ribotyping of *Lactobacillus casei* group strains isolated from dairy product. *Folia Microbiol.* 50, 223–228.
- Svec, P., Vancanneyt, M., Sedláček, I., Naser, S.M., Snauwaert, C., Lefebvre, K., Hoste, B., Swings, J., 2006. *Enterococcus silesiacus* sp. nov. and *Enterococcus termitis* sp. nov. *Int. J. Syst. Evol. Microbiol.* 56, 577–581.
- Tailliez, P., Tremblay, J., Ehrlich, S.D., Chopin, A., 1998. Molecular diversity and relationship within *Lactococcus lactis*, as revealed by randomly amplified polymorphic DNA (RAPD). *Syst. Appl. Microbiol.* 21, 530–538.
- Tamine, A.Y., Robinson, R.K., 1999. *Yoghurt Science and Technology*. CRC Press, Boca Raton.
- Temmerman, R., Scheirlinck, I., Huys, G., Swings, J., 2003. Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69, 220–226.
- Tilsala-Timisjarvi, A., Alatossava, T., 1997. Development of oligonucleotide primers from the 16S-23S rRNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. *Int. J. Food Microbiol.* 35, 49–56.
- Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K., 2001a. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* Species by RAPD-PCR and AFLP. *Syst. Appl. Microbiol.* 24, 554–560.
- Torriani, S., Felis, G.E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum*, by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Appl. Environ. Microbiol.* 67, 3450–3454.
- Torriani, S., Zapparoli, G., Dellaglio, F., 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. *Appl. Environ. Microbiol.* 65, 4351–4356.
- Tsakalidou, E., Zoidou, E., Pot, B., Wassill, L., Ludwig, W., Devriese, L.A., Kalantzopoulos, G., Schleifer, K.H., Kersters, K., 1998. Identification of streptococci from Greek Kasseri cheese and description of *Streptococcus macedonicus* sp. nov. *Int. J. Syst. Bacteriol.* 48, 519–27.

- Vancanneyt, M., Lombardi, A., Andrighetto, V., Knijff, E., Torriani, S., Bjorkroth, K.J., Fanz C. M.A.P., Foulquie Moreno, M.R., Revets, H., De Vuyst, L., Swings, J., Kersters, K., Dellaglio, F., Holzapfel, W.H., 2002. Intraspecies genomic groups in *Enterococcus faecalis* and their correlation with origin and pathogenicity. *Appl. Environ. Microbiol.* 68, 1381–1391.
- Vancanneyt, M., Naser, S.M., Engelbeen, K., De Wachter, M., Van der Meulen, R., Cleenwerck, I., Hoste, B., De Vuyst, L., Swings, J., 2006a. Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov. *Int. J. Syst. Evol. Microbiol.* 56, 1553–1557.
- Vancanneyt, M., Zamfir, M., De Wachter, M., Cleenwerck, I., Hoste, B., Rossi, F., Dellaglio, F., De Vuyst, L., Swings, J., 2006b. Reclassification of *Leuconostoc argentinum* as a later synonym of *Leuconostoc lactis*. *Int. J. Syst. Evol. Microbiol.* 56, 213–216.
- Vancanneyt, M., Huys, G., Lefebvre, K., Vankerckhoven, V., Goossens, H., Swings, J. (2006c). Intraspecific Genotypic Characterization of *Lactobacillus rhamnosus* Strains Intended for Probiotic Use and Isolates of Human Origin. *Appl. Environ. Microbiol.* 72, 5376–5383.
- Vasdinyei, R., Deak, T., 2003. Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. *Int. J. Food Microbiol.* 86, 123–130.
- Vasquez, A., Ahrne, S., Petterson, B., Molin, G., 2001. Temporal temperature gradient gel electrophoresis (TTGE) identification of *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus zae* and *Lactobacillus rhamnosus*. *Lett. Appl. Microbiol.* 32, 215–219.
- Ventura, M., Canchaya, Meylan, C., V., Klaenhammer, T.R., Zink, R. 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. *Appl. Environ. Microbiol.* 69, 6908–6922.
- Versalovic, J., Koeuth, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting bacterial genomes. *Nucl. Acids Res.* 19, 6823–6831.
- Versalovic, J., De Bruijn, M.S.F., Lupski, J.R., 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Meth. Mol. Cell. Biol.* 5, 25–40.
- Villani, F., Moschetti, G., Blaiotta, G., Coppola, S., 1997. Characterisation of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA. *J. Appl. Microbiol.* 82, 578–588.
- Ward, L.J.H., Timmins, M.J., 1999. Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. *Lett. Appl. Microbiol.* 29, 90–92.
- Zago, M., De Lorentiis, A., Carminati, D., Comaschi, L., Giraffa, G., 2006. Detection and identification of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophages by PCR. *J. Dairy Res.* 73, 146–53.
- Zamfir, M., Vancanneyt, M., Makras, L., Vaningelgem, F., Lefebvre, K., Pot, B., Swings, J., De Vuyst, L., 2006. Biodiversity of lactic acid bacteria in Romanian dairy products. *Syst. Appl. Microbiol.* 29, 487–495.
- Zhang, T., Fang, H.H.P., 2006. Applications of real-time polymerase reaction for quantification of microorganisms in environmental sample. *Appl. Microbiol. Biotechnol.* 70, 281–289.
- Zlotkin, A., Eldar, A., Ghittino, C., Bercovier, H., 1998. Identification of *Lactococcus garvieae* by PCR. *J. Clin. Microbiol.* 36, 983–985.

Chapter 3

Fermented Meat Products

Kalliopi Rantsiou and Luca Cocolin

Abstract The natural fermentation of Sausages is a complex microbial process in which the main participants are represented by lactic acid bacteria (LAB) and coagulase-negative cocci (CNC). The microflora of different types of fermented Sausages has been defined by using traditional microbiological methods based on isolation and biochemical identification. Since it appears that the types of microbial groups, or even the specific strains of a given microbial group, that dominate the fermentation, significantly affect the organoleptic profile of the final product, there is an increasing interest in the description of the microbiota that are found in different fermented Sausages. Recently new tools, based on molecular methods, allowing fast and unequivocal identification of strains, isolated from fermented Sausages, became available. These methods have been successfully applied and, in general, biochemical and molecular identification compared well. However, new information comes to light when molecular methods are applied to DNA and/or RNA extracted directly from Sausages. This approach eliminates problems related to traditional isolation. This chapter deals with the recent findings and results of the application of molecular methods, in a culture-dependent and culture-independent manner, on the study of the microflora of fermented Sausages.

1 Introduction

Meat fermentation is an ancient process originally used to extend the shelf life of perishable raw materials. During fermentation complex biochemical and physical reactions take place that result in a significant change of the initial characteristics. Moreover, production of aromatic substances during fermentation define the sensorial characteristics of the final product that are significantly different from the ones of the raw materials used.

The first evidence of Sausage production dates back to the period of the Roman empire (Lücke 1974).

Fermentation of Sausages is a well-known microbial process, and pioneering contributions on the ecology during ripening are available from the 1960s (Lerche and Reuter 1960; Reuter 1972; Lücke 1974). These studies stated that lactic

acid bacteria (LAB, *Lactobacillus* spp.) and coagulase-negative cocci (CNC, *Staphylococcus* and *Kocuria* spp.) are the two main groups of bacteria that are considered technologically important in the fermentation and ripening of Sausages.

LAB are usually present in high hygienic quality raw meat at low numbers ($10^2 - 10^3$ colony forming units, cfu/g), but they rapidly dominate the fermentation due to the anaerobic environment and the presence of NaCl, nitrate and nitrite, conditions that determine their selection. They reduce the pH of the Sausage with their main metabolic activity, the production of lactic acid from carbohydrates (Hammes, et al. 1990; Hammes and Knauf 1994). In addition to lactic acid production, LAB are responsible for the “tangy” flavor of Sausages, and for the small amounts of acetic acid, ethanol, acetoin, pyruvic acid and carbon dioxide (Demeyer 1982; Bacus 1986). Recently, it was demonstrated that *Lactobacillus* spp. commonly isolated from fermented Sausages possess proteolytic activity on muscle sarcoplasmatic proteins (Fadda, et al. 1998; Fadda, et al. 1999a; Fadda, et al. 1999b; Sanz, et al. 1999; Pereira, et al. 2001; Fadda, et al. 2002).

CNC participate in the development and stability of a generally appreciated red color through nitrate reductase activity that eventually leads to the formation of nitrosomyoglobin. Furthermore, nitrate reduction produces nitrite that can limit lipid oxidation (Talon, et al. 1999). By the activity of CNC, different aromatic substances and organic acids are also produced. In particular, proteolysis and lipolysis influence both texture and flavor development due to the release of low molecular weight compounds, including peptides, amino acids, aldehydes, amines and free fatty acids, which are important flavor compounds, or precursors of flavor compounds (Demeyer, et al. 1986; Schleifer 1986).

The type of microflora that develops in Sausage fermentation is often closely related to the ripening technique utilized. Sausage with a short ripening time have more lactobacilli from the early stages of fermentation, and an “acid” flavor predominates in the products, which are commonly sold after less than two weeks of ripening. The intensity of the flavor depends on the pH value, but at a given pH a high amount of acetic acid gives the product a less “pure” and more “sour” flavor (Montel, et al. 1998). Longer ripening times and greater activity of microorganisms other than LAB, such as CNC and yeasts, lead to higher levels of volatile compounds with low sensory thresholds. Lipids and peptides are precursors of most of these substances. Tissue enzymes are the main agents of initial lipolysis and proteolysis processes (Lücke 2000) however, later in aging, bacterial enzymes play a role in the degradation of peptides released (Molly, et al. 1997).

In some fermented Sausages, particularly those produced in France, Spain and Italy, the sensory properties of the products are also influenced by the development of the surface flora, consisting of molds and yeasts (Lücke 2000).

In naturally fermented Sausages there is an evident and strong connection between the microflora that develops during transformation and the sensory characteristics of the final product. As a consequence the study of the microbial ecology is an important parameter to consider in Sausage fermentation and for this reason is a subject of intense study.

The information collected so far on the ecology of fermented Sausages was obtained by using traditional microbiological methods, based on plating analysis and biochemical identification of isolated strains. With these techniques only easily culturable organisms are isolated and often microorganisms, for which selective enrichment and subculturing is problematic or impossible, cannot be characterized. The introduction of new approaches in the field of food microbiology exploiting molecular methods complements the studies carried out so far, and allows scientists to overcome the limitations of traditional methods. The developments in the study of the microbiota of naturally fermented Sausages as determined by molecular methods has been recently reviewed by Rantsiou and Cocolin (2006).

2 Culture-independent Methods

As mentioned above, conventional microbiological methods are not always suitable for isolation and characterization of microorganisms. In the last decade it was shown that classical microbiological techniques do not accurately detect microbial diversity (Hugenholtz, et al. 1998; ben Omar and Ampe 2000) and, as a consequence, an increasing interest in the development and use of culture-independent techniques was shown. New methods to directly characterize the microorganisms in particular habitats, without the need for enrichment or isolation, have been proposed (Head, et al. 1998). These approaches examine the total microbial DNA (or RNA) derived from mixed microbial populations to identify individual constituents (Hugenholtz and Pace 1996). In this way the necessity for strain isolation is eliminated, thereby negating the potential biases inherent to traditional culturing techniques. Studies, which employed such direct analysis, have often demonstrated a big variance between cultivated and naturally occurring species, thereby dramatically altering our understanding of the true microbial diversity present in various habitats (Cocolin, et al. 2002; Cocolin and Mills 2003).

One culture-independent method for studying the diversity of microbial communities in fermented Sausages is analysis of PCR products, generated with primers homologous to relatively conserved regions in the genome, by using denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) (Ercolini 2004). These approaches allow separation of DNA molecules that differ by a single base (Myers, et al. 1987; Muyzer, et al. 1995; Heuer and Smalla 1997) and, hence, have the potential to provide information about variations in target genes in a bacterial population. By adjusting the primers used for amplification, both major and minor constituents of microbial communities can be characterized. Moreover, modern image analysis systems have proven to be of value for the analysis of DGGE bands and their associated patterns. For instance, pairwise matching of DGGE bands in separate gel lanes has facilitated the calculation of similarity coefficients to describe relationships between communities (van der Gucht, et al. 2001).

A second culture-independent method used to study microbial ecology in fermented Sausages is represented by direct PCR and reverse transcription (RT)-PCR amplification using species-specific primers. The possibility to exploit the potential of a polymerase chain reaction to amplify, theoretically, a single nucleic acid molecule allows the detection of low-number populations that may be lost when traditional methods, such as plating or selective enrichments, are used. Species-specific PCR is a rapid and reliable molecular technique for the characterization of bacterial communities without colony isolation. However, the sensitivity of PCR in foods can be reduced due to the complexity of the food matrix and the presence of many PCR inhibitors. Many substances have been proven to be PCR inhibitory (Buffone, et al. 1991; Rossen, et al. 1992; Akane, et al. 1994; Powell, et al. 1994) and for this reason appropriate DNA and RNA extraction protocols, chosen on the basis of the food matrix under study, are used to avoid non-amplification of microbial species actually present in the ecosystem being investigated.

The papers describing the application of culture-independent methods in the definition of the microflora in fermented Sausages are reported in Table 3.1. As described above, the culture-independent methods are based on a direct extraction of DNA and/or RNA from the sample under investigation. The purified nucleic acids are subsequently subjected to molecular methods that are able to profile the bacterial populations present. The approaches that can be considered are either based on the use of molecular probes, or the use of PCR by itself or coupled with other techniques.

Despite the fact that specific probes have been developed for the identification of lactobacilli commonly isolated from meat (Hertel, et al. 1991; Nissen and Dainty 1995), there are no papers available on the direct application of these probes to target specific LAB directly in the Sausages during fermentation. The available data refer only to the rapid identification of isolated strains.

For the purpose of the direct profiling of the microflora in fermented Sausages the main protocols exploited were species-specific PCR and the PCR-DGGE method. It is important to underline that the direct approaches have only recently been applied to Sausage fermentations. Studies so far available on the direct profiling of Sausage microflora have been performed on Argentinean, Italian and Spanish fermented Sausages (Cocolin, et al. 2001a; Aymerich, et al. 2003; Rantsiou, et al. 2005b; Fontana, et al. 2005a; Fontana, et al. 2005b), and on fresh Sausages produced in Italy (Cocolin, et al. 2004).

Aymerich, et al. (2003) described the use of several species-specific primers to identify without traditional isolation and identification, LAB and CNC members in “chorizo” and “fuet,” two traditional fermented Sausages produced in Spain. The investigation focused on products at the end of maturation, and the experimental approach consisted of the amplification of DNA extracted from the sample, either subjected to an enrichment step or not. Six species of LAB and six species of CNC were targeted with specific primers. In particular, *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum*, *Enterococcus faecium*, *Lactococcus lactis*, *Pediococcus acidilactici*, *S. carnosus*, *S. warneri*, *K. varians*, *S. xylosum*, *S. simulans* and *S. epidermidis* were considered. Authors concluded that *Lb. sakei* and *S. xylosum* can be considered the predominant species in slightly fermented Sausages produced in Spain. Also *Lb. plantarum*,

Table 3.1 LAB and CNC Isolated from Naturally Fermented Sausages from Different Countries and Identified by Culture-independent Molecular Methods (The species are ordered on the basis of their prevalence in the product studied)

Country	Traditional product	Bacterial species	Identification method	Reference
Argentina	Tucuman Sausage	<i>Lb. sakei</i> <i>S. saprophyticus</i>	16S rRNA gene PCR-DGGE	Fontana, et al. 2005b
Italy	Salame friulano	<i>Lb. sakei</i> <i>Lb. curvatus</i> <i>S. xylosus</i> <i>S. intermedius</i> <i>S. carnosus</i> <i>S. lentus</i> <i>S. pulvereri</i> <i>Lb. plantarum</i>	16S rRNA gene PCR-DGGE	Cocolin, et al. 2001a
		<i>Lb. sakei</i> <i>Lb. curvatus</i> <i>S. xylosus</i> <i>Lb. plantarum</i>		
		<i>Lb. sakei</i> <i>Lb. curvatus</i> <i>Lb. paracasei</i>		
		<i>S. xylosus</i> <i>S. sciuri/pulvereri</i> ^a <i>S. equorum/succinus</i> ^a <i>L. garvieae</i> <i>S. intermedius</i> <i>M. caseolyticus</i>		
		<i>Lb. curvatus</i> <i>Lb. plantarum</i> <i>S. xylosus</i>		
		<i>Lb. sakei</i> <i>Lb. plantarum</i> <i>Lb. curvatus</i> <i>S. xylosus</i>		
Spain	Chorizo ^b	<i>Lb. sakei</i> <i>Lb. plantarum</i> <i>Lb. curvatus</i> <i>S. xylosus</i>	direct species-specific PCR	Aymerich, et al. 2003

^aThe method used did not allow a definitive identification.

^bResults obtained after an enrichment step at 30 °C for 24 h in MRS broth and in mannitol salt broth for LAB and CNC, respectively.

detected in 100 percent of “chorizos” and in only 50 percent of the “fuets,” should be considered an important agent of Spanish Sausage fermentation. This difference in the detection should be explained by the different technology used for the production of the two types of Sausages studied.

The use of the enrichment can be considered to introduce biases in the results obtained on the profiling of the microflora; in fact some populations can take over and inhibit the growth of numerically less important species. However, the use of selective or elective media in samples that will be subjected to DNA extraction and

PCR is a common practice in the field of food microbiology because it helps to overcome problems related to the PCR method. More specifically, with an enrichment step, target cells are increasing in number making their detection easier, only alive cells are amplified and, lastly, by diluting the sample in liquid enrichment media, PCR inhibitors are also diluted.

The disadvantage of a method based on species-specific probes for hybridization or species-specific primers for amplification is that, unavoidably, the number of species that may be detected is limited and it is lower or equal to the number of probes or sets of primers. These methods are applicable if we are interested in the dynamics of specific organisms. In this context, the development of a real-time PCR protocol able to quantify *Lb. sakei* in meat and fermented Sausages has recently been described (Martin, et al. 2006a). If the aim of the study is to obtain a more complete picture of the fermentation microflora and its evolution with time, alternative techniques are required.

The most suitable technique for the profiling of the microflora present in a specific environment should be able to detect, simultaneously, all the bacterial species present. This goal can be almost completely achieved with the application of the PCR-DGGE technique. Additionally, DGGE supports the species identification of community members because the amplification products, after they have been separated by DGGE, can be recovered from the gels and sequenced (Cocolin, et al. 2001a; Cocolin, et al. 2004; Mills, et al. 2002).

The first application of PCR-DGGE to profile the dynamic change during Sausage fermentation was described by Cocolin, et al. (2001a). Population successions were investigated as well as the species metabolically active during the process. In this study the V1 region of the 16S rRNA gene was selected for the amplification purpose. It has been recently reported that the use of the V1 region alone in DGGE analysis should be used carefully, because of its limited length (Yu and Morrison 2004). However, in the first study performed on DGGE profiling of fermented Sausage microflora it was concluded that only PCR products of the V1 region allow the differentiation between LAB and CNC involved in the transformation process. Products produced from the V3, V6-V8 and V9 regions of the 16S rRNA genes always resulted in co-migrations of control species of LAB and CNC used in the optimization of the protocol (Cocolin, et al. 2001a). In this study, both DNA and RNA were sampled directly to determine the levels of expression of the 16S rRNA gene of the most prominent bacteria, which may reflect their contributions to the fermentation process. The main difference detected by sampling RNA rather than DNA was the presence of natural meat contaminants, such as *Brochothrix thermosphacta*, *Enterococcus* sp., *Leuconostoc mesenteroides* and *Brevibacillus* sp., which disappeared after the third day. *Staphylococcus* species were found only in the meat mixture before Sausages were filled and after three days. The only *Staphylococcus* species represented in the DGGE gel after three days was *S. xylosus*, which produced a specific band until the end of fermentation. *Lb. sakei* and *Lb. curvatus* were the two species of LAB strongly present at both DNA and RNA levels from the third day of fermentation. One band, at DNA level, was identified as *Lb. plantarum*, but it was probably generated from dead cells

present in the meat, since no *Lb. plantarum* cells were isolated during fermentation and no specific signal was detected in the RNA amplicons.

Rantsiou, et al. (2005b) compared the microflora, as determined by PCR-DGGE, of three traditional fermented Sausages produced in three plants from the Northeastern part of Italy. The bacterial ecology was mainly characterized by the stable presence of *Lb. curvatus* and *Lb. sakei*, but *Lb. paracasei* was also repeatedly detected. Important evidence was the presence of *L. garvieae*, which clearly contributed in two fermentations. Several members of *Staphylococcus* were also detected. Regarding other bacterial groups *Bacillus* sp., *Ruminococcus* sp. and *Macroccoccus caseolyticus* were also identified at the beginning of the transformations (Fig. 3.1 and Table 3.2). In addition, yeast species belonging to *Debaryomyces hansenii*, several *Candida* species and *Willopsis saturnus* were observed in the DGGE gels (Fig. 3.2 and Table 3.3), and these results were also confirmed by a study on the yeast ecology of fermented Sausages conducted by Cocolin, et al. (2006b). The dominance of *D. hansenii* was determined in the DGGE gels, where no other yeast band could be detected. In this study, molds, such as *Penicillium farinosum*, *Penicillium viridicatum* and *Mucor racemosus*, were observed as well. Cluster analysis of the DGGE profiles highlighted how the three fermentations shared different levels of similarity, when bacterial and yeast ecologies were considered. Samples analyzed in the early stages of fermentations were more diverse, and they formed single clusters (Fig. 3.3).

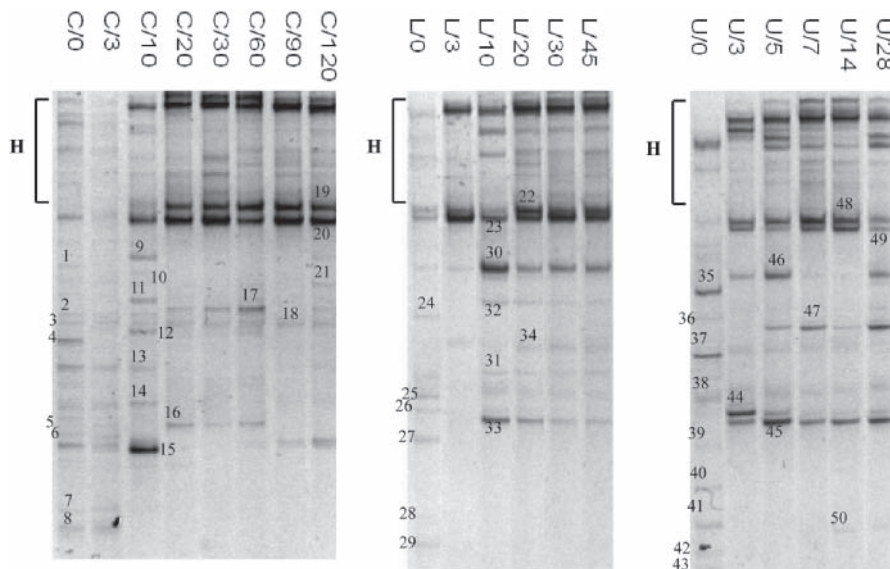


Fig. 3.1 Bacterial DGGE profiles of three fermented Sausages during ripening. Lane designations indicate the plant code (C, L, U) and the day of sampling. The gels shown are not normalized. Bands indicated by numbers were excised and, after re-amplification, subjected to sequencing. H, heteroduplex bands (reprinted by permission from Rantsiou, et al. 2005b)

Table 3.2 Sequencing Results of Bands Cut from Bacteria-DGGE Gels Analyzing Fermented Sausages Ecology (reprinted by permission from Rantsiou, et al. 2005b)

Bands ^a	Size (bp)	Closest relative	%Identity	Source ^b
1	113	<i>Lactobacillus sakei</i>	99.1	AB124845
2	91	<i>Staphylococcus xylosus</i>	98.9	AY126259
3, 18, 24, 36	113	<i>Lactobacillus curvatus</i>	99.6	AY204894
4	75	Bacterium Ellin5218	100	AY234569
5, 25, 38	91	<i>Staphylococcus sciuri/pulvereri</i> ^c	98.9	AY126231
				AY126216
6	91	<i>Staphylococcus equorum/succinicus</i>	98.2	AF527483
				AY126240
7, 41	93	<i>Macrococcus caseolyticus</i>	96.7	AY126157
8	91	<i>Bacillus</i> sp.	98.8	AY461682
9	91	<i>Staphylococcus xylosus</i>	98.6	AY126259
10, 30, 46	93	<i>Lactococcus garvieae</i>	99.6	AY438044
11	91	<i>Staphylococcus xylosus</i>	97.9	AY126259
12, 47	91	<i>Staphylococcus xylosus</i>	98.9	AY126259
13, 37	91	<i>Staphylococcus equorum/succinicus</i> ^d	98.7	AF527483
				AY126240
14	91	<i>Staphylococcus xylosus</i>	99.0	AY126259
15, 33, 45	91	<i>Staphylococcus equorum/succinicus</i>	98.9	AF527483
				AY126240
16, 26, 44	111	<i>Lactobacillus paracasei</i>	99.0	AY204894
17, 32	111	<i>Lactobacillus curvatus</i>	99.1	AY204894
19, 22, 48	111	<i>Lactobacillus curvatus</i>	97.2	AY204894
20, 23, 49	113	<i>Lactobacillus sakei</i>	99.5	AB124845
21, 35	91	<i>Brochotrix thermosphacta</i>	98.9	M58798
27, 39	93	<i>Staphylococcus intermedius</i>	100	D83369
28	91	<i>Bacillus</i> sp.	98.8	AY461682
29	106	<i>Ruminococcus</i> sp.	100	AY442822
31	114	<i>Lactobacillus sakei</i>	98.2	AB124845
34	93	<i>Lactococcus garvieae</i>	98.9	AY438044
41, 50	93	<i>Macrococcus caseolyticus</i>	98.9	AY126157
42	95	<i>Macrococcus caseolyticus</i>	97.7	AY126157
43	91	<i>Bacillus pumilis</i>	98.8	X60637

^aBands numbered as indicated on DGGE gels shown in Fig. 3.1.

^bAccession number of sequence of closest relative found with Blast search.

^{c,d}The V1 regions were 100 percent identical not allowing identification.

Concerning the yeast ecology, the fermentations showed a specific yeast pattern. On the basis of these results, it was concluded that both bacterial and yeast ecologies were characteristic of each fermentation. A high level of information could be obtained by the application of molecular methods. The importance of species commonly recognized as responsible for Sausage fermentation (e.g., *Lb. sakei* and *Lb. curvatus*) was confirmed. In addition, for the first time, evidence was obtained showing that strains belonging to *L. garvieae* and *M. caseolyticus*, that are

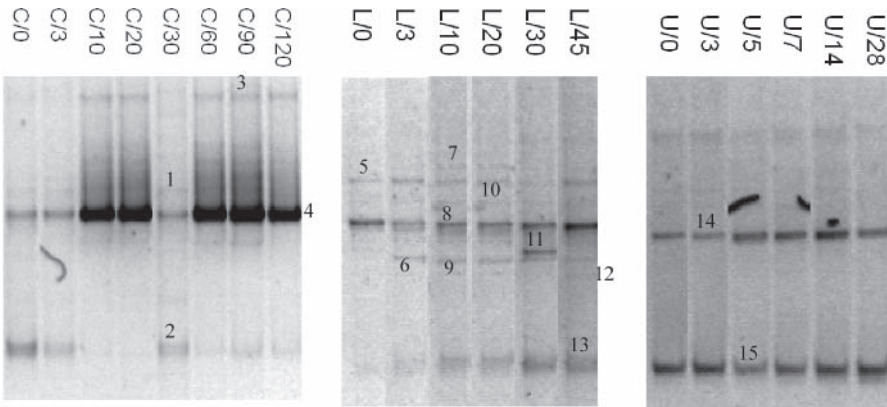


Fig. 3.2 Yeast DGGE profiles of three fermented Sausages during ripening. Lane designations indicate the plant code (C, L, U) and the day of sampling. The gels shown are not normalized. Bands indicated by numbers were excised and after re-amplification subjected to sequencing (reprinted by permission from Rantsiou, et al. 2005b)

Table 3.3 Sequencing Results of Bands Cut from Yeast-DGGE Gels Analyzing Fermented Sausages Ecology (reprinted by permission from Rantsiou, et al. 2005b)

Band ^a	Size (bp)	Closest relative	%Identity	Source ^b
1	241	<i>Candida tropicalis</i>	97.5	AF267497
2, 13, 15	246	<i>Mayaca fluviatilis</i>	86.5	AF293855
3	249	<i>Debaryomyces hansenii</i>	99.3	AF485980
4	249	<i>Debaryomyces hansenii</i>	99.3	AF485980
5	242	<i>Candida krisii</i>	100	AJ539355
6	248	<i>Willopsis saturnus</i>	99.6	AJ507804
7, 10	247	<i>Aerobasidium pullulans</i>	100	AY185811
8	248	<i>Willopsis saturnus</i>	100	AJ507804
9	248	<i>Candida fermentati</i>	100	AY187283
10	248	<i>Willopsis saturnus</i>	99.8	AJ507804
12	203	<i>Entyloma dahliae</i>	100	AY272033
14	242	<i>Candida sake/austromarina</i> ^c	98.9	AJ549822
				U62310

^aBand numbered as indicated on DGGE gels shown in Fig. 3.2.
^bAccession number of sequence of closest relative found with Blast search.
^cThe D1-D2 loop regions were 100 percent identical not allowing identification.

commonly isolated from dairy products, may also be involved in Sausage fermentation. Since milk powder and/or lactose are commonly used in the production of fermented Sausages, it can be assumed that these microorganisms come from these sources. However, the Sausages studied by Rantsiou, et al. (2005b) did not contain these ingredients in their formula.

PCR-DGGE was also used to monitor the dynamics of bacteria in Argentinean dry fermented Sausages (Fontana, et al. 2005a; Fontana, et al. 2005b). In these

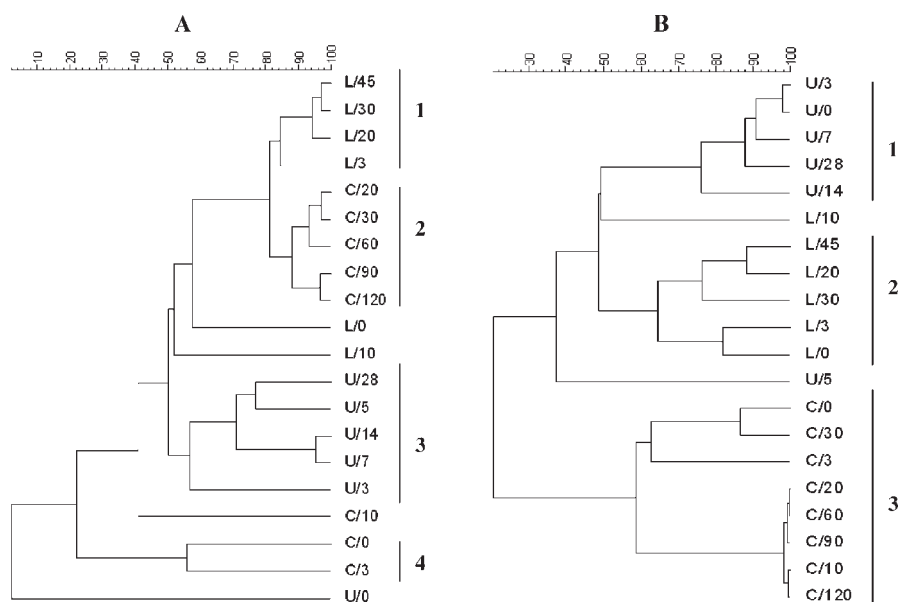


Fig. 3.3 Cluster analysis of the profiles obtained by bacterial (A) and yeast (B) DGGE analysis of three fermented Sausages. Dendrograms were obtained with the UPGMA clustering algorithm. Samples are indicated by the plant code (C, L, U) and the day of analysis. Identified clusters are indicated by numerals to the right of each panel (reprinted by permission from Rantsiou, et al. 2005b)

studies the capability of two primers commonly used in DGGE analysis to differentiate LAB and CNC isolated from Tucuman Sausages was evaluated. Primers Bact-0124f(GC)-Uni-0515r and V1f(GC)-V1r were used and it was determined that both allowed the study of the succession of different *Lactobacillus* and *Staphylococcus* species during the ripening process. *Lb. sakei* was shown to be pre-dominant in the samples considered. *S. saprophyticus* was only observed in “Tucuman Sausage” while a band identified as *B. thermosphacta* was detected in “Cordoba Sausages” (Fontana, et al. 2005a).

An interesting application of PCR-DGGE in the field of fermented Sausages was also described by Cocolin, et al. (2006a). In this study DGGE was used as a tool to follow *Lb. plantarum* and *S. carnosus* inoculated as a starter culture in fermented Sausage production. While the capability of the *Lb. plantarum* strain to conduct the fermentation was demonstrated, only a marginal contribution was observed for *S. carnosus*. Moreover, an intense band identified as *Lb. curvatus* was present throughout the fermentation. This species, most probably coming from the meat or the production environment, was only marginally isolated from the plates, but the PCR-DGGE analysis conducted at both DNA and RNA levels, highlighted its importance in this specific Sausage production.

The application of the 16S rRNA gene PCR-DGGE analysis is not, however, free of problems. One hurdle to overcome is the sensitivity of the method for the

detection of low number populations. Cocolin, et al. (2001a) defined that, in mixed populations, individual members could be identified by PCR-DGGE of the V1 region when the concentrations are more than 10^4 cfu/g, which allows detection of species at a threshold level during fermentation. This may not be true when other primers are applied in the PCR amplification step. As a consequence, special attention should be dedicated to this issue to carefully profile the microflora present in specific ecosystems. A second problem, related to the technique when rRNA amplicons are analyzed by DGGE, is the presence of multiple copies of the ribosomal genes. Due to its heterogeneity, several bands per species can be seen in high-resolution PCR-DGGE analysis. The amplified fragments will, therefore, appear as several bands on a DGGE gel, rather than a single band that would allow precise species identification.

A possible solution to the heterogeneity of the 16S rRNA gene was given by Dahllöf, et al. (2000), which proposed the *rpoB* gene, coded for the β subunit of the RNA polymerase, as a target for PCR-DGGE. The approach resulted to be convenient for environmental samples (Dahllöf, et al. 2000), and it was further applied to food fermentations, including fermented Sausages (Rantsiou, et al. 2004). In this study, the contribution of *Lb. sakei*, *Lb. curvatus* and *S. xylosus* was again pointed out. Interestingly, a band belonging to *Lb. plantarum* was constantly present after the tenth day of fermentation.

Lastly, the incomplete extension of the GC clamp during PCR amplification, which may result in artifactual double bands in DGGE analysis, often complicates the interpretation of the profiles. This issue was recently addressed by Janse, et al. (2004) and it was proposed that it can be eliminated by increasing the time of the final extension step of the PCR reaction.

3 Culture-dependent Methods

The study of the fermented Sausage microflora by traditional methods has been carried out by different authors in the last 20 years. In some cases, only the technologically relevant microbial groups were examined (LAB and CNC), whereas other studies reported the dynamics of several microbial populations, thereby carefully profiling the changes in the microbial ecology during fermentation and ripening. In the case of fermented Sausages, it has been defined that the de Man, Rogosa, Sharpe (MRS, de Man, et al. 1960) medium is the most suitable for the isolation of LAB, while for the CNC the Mannitol Salt Agar (MSA) (APHA, 1966) is often used due to its characteristics of selectivity towards halotolerant species. LAB and total aerobic microbes are the fastest growing during the production of Sausages. From the initial counts of 10^2 – 10^3 cfu/g, they reach values of 10^7 – 10^8 cfu/g in the first three days of fermentation (Sanz, et al. 1988; Samelis, et al. 1993; Rebecchi, et al. 1998; Samelis, et al. 1998; Cocolin, et al. 2001a; Cocolin, et al. 2001b; Metaxopoulos, et al. 2001; Papamanoli, et al. 2002; Papamanoli, et al. 2003; Aymerich, et al. 2003; Mauriello, et al. 2004; Drosinos, et al. 2005; Comi, et al.

2005), and this situation establishes both in the core and in the external layers of the Sausage (Coppola, et al. 2000). Their counts remain quite stable throughout the ripening period. Also the CNC show a rapid increase in the first days of fermentation, but in some cases it has been reported that fast growth of LAB, with the consequence of deep acidification of the substrate, could result in inhibitions towards CNC, that exhibit a slow growth (Papamanoli, et al. 2002; Papamanoli, et al. 2003). However, in several studies it was shown that CNC, together with LAB, are the populations numerically more important at the end of the ripening period (Rebecchi, et al. 1998; Cocolin, et al. 2001a; Aymerich, et al. 2003; Mauriello, et al. 2004). Only in few cases were yeasts found to be relevant to Sausage fermentation. Samelis, et al. (1993) determined a stable number of 10^5 cfu/g in all stages of production, and this data was confirmed by Metaxopoulos, et al. (2001). Coppola, et al. (2000) found yeasts to be a predominant flora, together with LAB and CNC, in Naples-type salami, with *Debaryomyces* spp., described as a proteolytic agent during fermentation of Sausages (Santos, et al. 2001), as the main representative. Recently, Cocolin, et al. (2006b) studied the yeast dynamics during fermentation of Sausages conducted at low temperatures (10°C). Counts above 10^6 cfu/g were found for almost all of the maturation period. *D. hansenii* was the main species isolated, but *C. zeylanoides* and *Pichia triangularis* were detected as well. Significant differences on the presence and persistency of enterococci have been reported by different authors. In some studies, after an increase to 10^5 cfu/g up to the 20 days of fermentation, a reduction in the numbers was observed, leading to a final count of about 10^2 cfu/g (Cocolin, et al. 2001a; Cocolin, et al. 2001b; Aymerich, et al. 2003; Papamanoli, et al. 2003). On the other hand, several papers reported a stable population of enterococci of about 10^5 – 10^6 cfu/g at the end of the fermentation (Rebecchi, et al. 1998; Samelis, et al. 1998; Metaxopoulos, et al. 2001; Comi, et al. 2005; Rantsiou, et al. 2005b), becoming an important population possibly influencing the final organoleptic characteristics of the product. Since enterococci are able to produce ammonia and other amines, they possibly contribute to the final flavor of the product. Despite the concern about pathogenicity of enterococci, recent studies point out that food and meat enterococci – especially *E. faecium* – have a much lower pathogenicity potential than clinical strains. Enterococci possess a competitive advantage over other microbiota in meat fermentations, and many enterococci isolated from Sausages have the ability to produce enterocins harboring antimicrobial activity against pathogens and spoilage microorganisms of meat concern (Hugas, et al. 2003).

Total enterobacteria and *Escherichia coli* are usually counted only in the first days of fermentation and their number decreases due to the acidification performed by LAB (Rebecchi, et al. 1998; Cocolin, et al. 2001a; Cocolin, et al. 2001b; Metaxopoulos, et al. 2001; Aymerich, et al. 2003; Papamanoli, et al. 2003; Drosinos, et al. 2005). Fermented Sausages are usually free of sulphite-reducing clostridia and coagulase-positive staphylococci (Samelis, et al. 1998; Cocolin, et al. 2001a; Cocolin, et al. 2001b; Papamanoli, et al. 2003; Aymerich, et al. 2003; Comi, et al. 2005; Drosinos, et al. 2005; Rantsiou, et al. 2005b), but in some studies suspected

colonies of *S. aureus* were isolated from the meat mix (Rebecchi, et al. 1998; Metaxopoulos, et al. 2001).

Considering the safety aspect of the fermented Sausages, several studies investigated the presence of *Listeria monocytogenes* and *Salmonella* spp. during the different stages of the production. It has been defined that, generally, these food-borne pathogens are absent in 25 g of product (Aymerich, et al. 2003; Comi, et al. 2005; Rantsiou, et al. 2005b). *Listeria monocytogenes* can contaminate the fresh Sausage mix, but commonly it is undetectable at the end of the fermentation (Samelis, et al. 1998; Metaxopoulos, et al. 2001; Drosinos, et al. 2005).

4 Molecular Identification of Strains Isolated from Fermented Sausages by Culture-dependent Methods

For the purpose of identification of isolates by molecular methods, the gene more often targeted is the 16S rRNA. It possesses some key attributes that make it suitable for bacterial identification. In particular, it is common in all bacteria, it possesses variable and conserved regions that can be used for differentiation purposes and, lastly, it functions as an evolutionary clock, allowing for conclusions to be drawn regarding phylogeny (Woese 1987; Collins, et al. 1991; Gürtler and Stanisich 1996; Nour 1998). Moreover, a large database of sequences is already available.

The culture-dependent methods used so far in the field of fermented Sausages refer to the molecular identification and characterization of isolated LAB and CNC strains (Table 3.4). They are reproducible, easily automated and rapid (Charteris, et al. 1997). The increasing availability of the sequences of the 16S rRNA gene (Collins, et al. 1991) and the intergenic region between 16S rRNA and 23S rRNA genes (Nour 1998) allowed the development of different methods for the identification of microbial species of interest in the field of Sausage fermentation.

Ribosomal RNA probes (Nissen and Dainty 1995; Hertel, et al. 1991), species specific PCR primers (Berthier and Ehrlich 1998; Yost and Nattress 2000; Rossi, et al. 2001; Blaiotta, et al. 2003b; Corbiere Morot-Bizot, et al. 2003), randomly amplified polymorphic DNA (RAPD)-PCR analysis (Berthier and Ehrlich 1999; Andrigetto, et al. 2001), restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Sanz, et al. 1998; Lee, et al. 2004), multiplex PCR (Corbiere Morot-Bizot, et al. 2004), ribotyping (Zhong, et al. 1998), PCR amplification of repetitive bacterial DNA elements (rep-PCR) (Gevers, et al. 2001), TGGE (Cocolin, et al. 2000) and DGGE (Cocolin, et al. 2001b; Ercolini, et al. 2001; Blaiotta, et al. 2003a) have been applied for the identification of LAB and CNC isolated from fermentation of Sausages. Moreover, other molecular techniques were described as valuable tools for the same purposes. Samelis, et al. (1995) developed a sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis of whole-cell proteins to differentiate between *Lb. sakei* and *Lb. curvatus*, isolated from naturally fermented Greek dry salami, and Di Maria, et al. (2002) used pulsed field gel electrophoresis

Table 3.4 LAB and CNC Isolated from Naturally Fermented Sausages from Different Countries and Identified by Culture-dependent Molecular Methods (The species are ordered on the basis of their prevalence in the product studied)

Country	Traditional product	Bacterial species	Identification method	Reference
Argentina	N.S.	<i>Lb. sakei</i>	RAPD analysis and 16S rRNA gene sequencing	Fontana, et al. 2005a
		<i>Lb. plantarum</i>		
		<i>S. saprophyticus</i>		
France	N.S.	<i>Lb. sakei</i>	Species-specific PCR	Ammor, et al. 2005
		<i>E. faecium</i>		
		<i>L. garvieae</i>		
		<i>V. carniphilus</i>		
Greece	N.S.	<i>Lb. curvatus</i>	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou, et al. 2005a
		<i>Lb. sakei</i>		
		<i>Lb. plantarum</i>		
		<i>Lb. casei/paracasei</i> ²⁰		
		<i>Lb. paraplantarum</i>		
		<i>Lb. sakei</i>		
Hungary	N.S.	<i>Lb. curvatus</i>	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou, et al. 2005a
		<i>W. viridescens</i>		
		<i>W. paramesenteroides/hellenica</i> ²¹		
		<i>Lc. mesenteroides</i>		
Italy	N.S.	<i>Lb. sakei</i>	RAPD analysis and 16S rRNA gene sequencing	Rebecchi, et al. 1998
		<i>Lb. plantarum</i>		
	Salame friulano	<i>Lb. sakei</i>	PCR-TGGE	Cocolin, et al. 2000
		<i>Lb. casei</i>		
		<i>Lb. curvatus</i>		
		<i>Lb. alimentarius</i>		
	Salame friulano	<i>S. xylosum</i>	PCR-DGGE	Cocolin, et al. 2001b
		<i>S. carnosus</i>		
		<i>S. simulans</i>		
		<i>K. varians</i>		

(continued)

Table 3.4 LAB and CNC Isolated from Naturally Fermented Sausages from Different Countries and Identified by Culture-dependent Molecular Methods (The species are ordered on the basis of their prevalence in the product studied) (continued)

Country	Traditional product	Bacterial species	Identification method	Reference
Italy	Soppressata	<i>Lb. sakei</i>	RAPD-PCR	Andrighetto, et al. 2001
	Salame tradizionale	<i>Lb. curvatus</i>		
	Salsiccia sotto sugna	<i>S. xylosteus</i> <i>S. condimentii</i>	16S-23S rRNA genes intergenic region PCR	Rossi, et al. 2001
	N.S.	<i>S. xylosteus</i>	16S rRNA gene sequencing intergenic spacer region PCR	Blaiotta, et al. 2004
		<i>S. pulvererivitulius^a</i>		
		<i>S. equorum</i>	PCR-DGGE species-specific PCR	
		<i>S. saprophyticus</i>		
	Sardinian sausage	<i>Lb. sakei</i>	Species-specific PCR	Greco, et al. 2005
		<i>Lb. plantiarum</i>		
		<i>Lb. curvatus</i>		
	Salame friulano	<i>Lb. sakei</i>	PCR-DGGE and 16S rRNA gene sequencing	Rantsiou, et al. 2005a
		<i>Lb. curvatus</i>		
		<i>Lb. plantiarum</i>		
		<i>Lb. paraplantarum</i>		
	Salame friulano	<i>W. paramesenteroides/hellenica^a</i>		
		<i>Lb. sakei</i>	PCR-DGGE and 16S rRNA gene sequencing	Comi, et al. 2005
		<i>Lb. curvatus</i>		
		<i>Lb. plantiarum</i>		
		<i>Lb. paraplantarum</i>		
	Salame friulano	<i>Lb. sakei</i>	PCR-DGGE and 16S rRNA gene sequencing	Urso, et al. 2006
		<i>Lb. curvatus</i>		
		<i>Lb. plantiarum</i>		
		<i>Lb. casei</i>		
		<i>W. hellenica</i>		

(continued)

Table 3.4 LAB and CNC Isolated from Naturally Fermented Sausages from Different Countries and Identified by Culture-dependent Molecular Methods (The species are ordered on the basis of their prevalence in the product studied) (continued)

Country	Traditional product	Bacterial species	Identification method	Reference
Italy	Salame di Senise	<i>Lb. sakei</i>	RAPD-PCR and 16S rRNA gene sequencing	Baruzzi, et al. 2006
		<i>Lb. curvatus</i>		
		<i>Lb. casei</i>		
		<i>S. equorum</i>		
		<i>S. saprophyticus</i>		
		<i>S. succinus</i>		
Spain	Salame friulano	<i>S. xylosum</i>	Species-specific PCR	Iacumin, et al. 2006
		<i>S. warneri</i>		
		<i>S. pasteurii</i>		
	Chorizo, fuet, salchichon	<i>S. equorum</i>	Species-specific PCR	Aymerich, et al. 2006
		<i>Lb. sakei</i>		
		<i>Lb. curvatus</i>		
	Chorizo, fuet, salchichon.	<i>Lc. mesenteroides</i>	Species-specific PCR	Martin, et al. 2006b
		<i>S. xylosum</i>		
		<i>S. warneri</i>		
		<i>S. epidermidis</i>	Species-specific PCR	
		<i>S. carnosus</i>		
		<i>K. varians</i>		

(PFGE) to monitor *S. xylosus* DSM 20266 added as a starter during fermentation and ripening of “soppressata molisana,” a typical Italian Sausage.

4.1 *Lactic Acid Bacteria*

Despite the large number of molecular techniques developed for the identification of LAB isolated from fermented Sausages, only some were applied to define the microflora of these products. The studies that exploited molecular methods for this purpose are reported in Table 3.4. The first paper applying molecular methods for the identification of LAB in fermented Sausages was published in 1998 by Rebecchi, et al. They used RAPD analysis to group the isolated LAB, which were subsequently subjected to the 16S rRNA gene sequencing. The authors reported that the isolates could be grouped in few groups with the same RAPD pattern, and after sequencing they belonged to *Lb. sakei* and *Lb. plantarum*. Moreover, it was demonstrated that the ripening process of Italian dry Sausage is driven by a limited number of strains of lactobacilli due to their ability to compete best under the prevailing conditions of the niche. The RAPD-PCR approach was also used by Andrighetto, et al. (2001) that identified *Lb. sakei* and *Lb. curvatus* from traditional salami produced in the Veneto region of Italy.

The larger part of the scientific literature regarding molecular methods applied to the identification and characterization of LAB strains was produced in the last five years. Versatile methods, recently extensively used for strain identification, are DGGE and TGGE. Since the separation between strains is based on differential migrations, these techniques can be used for screening and grouping the isolates, thereby reducing the number of cultures to identify by 16S rRNA gene sequencing. In 2000 Cocolin, et al. published a paper focusing on the development of TGGE to identify LAB strains isolated from naturally fermented Sausages. The PCR-TGGE method allowed differentiation of *Lb. brevis*, *Lb. curvatus*, *Lb. alimentarius*, *Lb. casei*, *Lb. plantarum* and *Lb. sakei*. These lactobacilli were characterized by different migration patterns. Thirty-nine strains were subjected to PCR-TGGE analysis and the results obtained underlined that the majority of the strains belonged to *Lb. sakei* (37 percent) and *Lb. casei* (34 percent), while *Lb. curvatus* and *Lb. alimentarius* were isolated less frequently (18 percent and 11 percent, respectively). A similar approach employing DGGE and subsequent sequencing of representative strains, was used to identify LAB during production of Italian, Greek and Hungarian fermented Sausages (Cocolin, et al. 2000; Comi, et al. 2005; Rantsiou, et al. 2005a). It is interesting to note that the application of PCR-DGGE and 16S rRNA gene sequencing allows quick identification of a large number of strains.

Species specific PCR has been used often to obtain a fast and reliable identification of isolated strains from fermented Sausages. Greco, et al. (2005) used this method to study the evolution of LAB in Sardinian Sausages. *Lb. sakei*, *Lb. plantarum* and *Lb. curvatus* were the main species isolated during fermentation and maturation.

The same approach for LAB identification was also used later by other authors to elucidate the dynamics of LAB strains isolated from a small-scale facility producing traditional dry Sausages (Ammor, et al. 2005). In this study it was determined that the LAB ecosystem is characterized by a species-site-dominance. This dominance is closely related to the environmental parameters. While it is important in raw materials and in processing materials, it decreased with in the setting of fermentation process when *Lb. sakei* became the predominant species in the fermented product. Interestingly, other species, such as *E. faecium*, *L. garvieae*, *Vagococcus carniphilus* and *Lc. mesenteroides*, were identified as well.

The approach of species-specific PCR, coupled with RAPD analysis for strains characterization, was used to define the ecology of LAB in Italian fermented Sausages (Comi, et al. 2005, Urso, et al. 2006), to investigate the diversity of LAB isolated in Greece, Hungary and Italy (Rantsiou, et al. 2005a), and to type LAB strains from slightly fermented Sausages (Aymerich, et al. 2006).

Comi, et al. (2005) studied naturally fermented Sausages produced in the Northeast of Italy and used species-specific PCR, DGGE, 16S rRNA gene sequencing and RAPD analysis for the identification and characterization of 150 LAB isolates during three fermentation processes. Once more, *Lb. sakei* and *Lb. curvatus* represented the two species most often isolated, accompanied by low numbers of other lactobacilli, such as *Lb. plantarum* and *Lb. paraplantarum*, *Leuconostoc* and *Weissella* spp. RAPD analysis carried out on the *Lb. sakei* and *Lb. curvatus* strains underlined the intraspecies diversity. It is highly probable that strains that grouped in the fermentation-specific clusters came from the ingredients used in the production. Moreover, it may be assumed that the slight differences in the sensory characteristics of the products were due, at least partially, to the presence of different *Lb. sakei* and *Lb. curvatus* strains, as determined by RAPD-PCR cluster analysis.

The use of RAPD analysis for strain characterization was also described by Rantsiou, et al. (2005a) with the specific goal of understanding the diversity of LAB strains in three different countries of Europe. In this study, 358 LAB strains were isolated during production of Greek, Hungarian and Italian naturally fermented Sausages. After identification by molecular methods, RAPD analysis identified clusters based on the provenience of the strains. *Lb. plantarum*, *Lb. curvatus* and *Lb. sakei*, that were common to all the fermentations followed, grouped in country-specific clusters mainly constituted by isolates coming from one country with a few others from one or both the other countries considered in the study.

New information from applying RAPD analysis was also reported by Urso, et al. (2006). While the approach for the LAB isolates identification was again based on DGGE grouping followed by 16S rRNA gene sequencing, the use of RAPD allowed understanding of the strain dynamics during fermentation. After cluster analysis of *Lb. sakei* isolated during transformation, it was possible to highlight which RAPD-type strain was able to take over and conduct the fermentation studied. In the case of the three plants followed in this study, only in one, two-RAPD type strains were equally represented during processing, while in the other two, only one strain was able to dominate the fermentation process.

Lastly, RAPD analysis and plasmid profiling were exploited by Aymerich, et al. (2006) to type LAB isolated from slightly fermented Sausages in Spain. Combining the typing methods, it was possible to differentiate 144 strains out of 250 isolates. *Lc. mesenteroides* showed the higher intraspecific variability, since it was possible to differentiate nine profiles out of the 12 isolates of this species, while *Lb. sakei* showed 112 different strains out of 185 isolates. *Lb. curvatus* presented lower-intraspecific variability than the other species.

4.2 Coagulase Negative Cocci

As with LAB, the molecular approaches for CNC were exploited mainly for strain identification and characterization (Table 3.4). One of the first studies proposed for the identification of CNC isolated from fermented Sausages by PCR-DGGE was published by Cocolin, et al. (2001b). While at the beginning of the fermentation strains of *S. simulans*, *S. carnosus* and *K. varians* were identified, from day 10 only *S. xylosus* could be detected. The CNC ecology was represented by this species until day 45. The PCR-DGGE method was also used by other authors for strain identification. Ranstiou, et al. (2005c) used this method to identify 85 strains of CNC isolated from fresh Sausages. Almost 50 percent of the isolates were identified as *S. xylosus*, but strains of *S. pasteurii*, *S. warneri*, *S. equorum* and *S. succinus* were also found. Moreover, Iacumin, et al. (2006b) coupled the DGGE method with 16S rRNA gene sequencing to identify 617 strains of CNC isolated from three different plants during the fermentation process. The same species of CNC were found in all three processing plants, but their contribution to the fermentations was different. In two plants *S. xylosus* was the main species involved in the fermentation process, while in the third the maturation was carried out equally by three species: *S. xylosus*, *S. warneri* and *S. pasteurii*.

Blaiotta, et al. (2004a) exploited several molecular methods, such as 16S rRNA gene sequencing, species-specific PCR assays, intergenic spacer region-PCR and PCR-DGGE, in order to identify 471 strains of CNC isolated from traditionally fermented Sausages produced in Basilicata in Southern Italy. The CNC microflora was found to be dominated by different biotypes of *S. xylosus*, followed by *S. pulvereri/vitulis*, *S. equorum* and *S. saprophyticus*. Other species were also present, but at lower levels and 25 percent of the isolates could not be identified by the methods used.

Other methods based on 16S–23S rRNA genes intergenic PCR and species-specific PCR were used as well, for the identification of CNC (Rossi, et al. 2001; Blaiotta, et al. 2003b). Moreover, Blaiotta, et al. (2004b; 2005) designed specific primers on the *sodA* gene for the identification of *Staphylococcus* species isolated from fermented Sausages.

As already described for LAB, there is a large literature on the use of molecular typing methods for strain characterization for CNC. These studies focus mainly on isolates from different Sausages produced in Italy and Spain, but the staphylococcal

community of a small unit manufacturing traditional dry fermented Sausage in France was also investigated in two different seasons (Corbiere Morot-Bizot, et al. 2006). In this study a collection of 412 *Staphylococcus* isolates was studied and multiplex PCR, PFGE and sequencing of the *sodA* gene were used to identify and characterize the isolates. The combination of the molecular methods allowed the identification of seven species of *Staphylococcus* in spring and five in winter. *S. equorum* and *S. succinus* dominated both the environment and the meat products. They represented 49 percent and 33 percent of the isolates, respectively. PFGE allowed the assignment of *S. equorum* to eight pulsotypes showing a wide diversity among this species. But the entire environment and the meat products were dominated by one pulsotype. For *S. succinus*, three pulsotypes were found with one dominant mainly isolated during the spring sampling. This study highlighted the diversity of staphylococci isolated in the environment and in the meat products of a small processing unit manufacturing traditional dry fermented Sausages.

RAPD methods were also used for characterization of CNC populations isolated from slight fermented Sausages (Martin, et al. 2006b), and for genotyping clustering of *S. xylosus* obtained from Italian artisanal Sausages “salsiccia sotto sugna” (Rossi, et al. 2001) and “salame friulano” (Iacumin, et al. 2006a).

Rossi, et al. (2001) subjected *S. xylosus* to RAPD-PCR using primers OPL-1, OPL-2, OPL5 and Hpy 1 and after cluster analysis they were able to separate strains showing nitrate reduction and amino acid decarboxylase activities, thereby discriminating strains of *S. xylosus* with relevant technological activities.

Lastly, Iacumin, et al. (2006a) performed a study on the molecular and technological characterization of *S. xylosus* isolated from naturally fermented Italian Sausages produced in three different processing plants in the Friuli Venezia Giulia region in Northeast Italy. Two-hundred-forty-nine strains of *S. xylosus* were identified by species-specific PCR and subjected to molecular and technological characterization. RAPD-PCR with primer M13, Rep-PCR using primer (GTG)₅ and Sau-PCR with primer SAG₁ were used for the molecular analysis, while the capability of the strains to grow at different temperatures and in the presence of NaCl and their lipolytic and proteolytic activity were tested in order to define the technological characteristics. The results obtained allowed the differentiation of strains coming from different plants, and the recognition that these strains are plant-specific.

The picture of the fermented Sausage microflora that can be observed with the application of molecular methods does not disagree with the one produced using biochemical identification. Once more the predominance of *Lb. sakei* and *Lb. curvatus*, and of *S. xylosus*, is emerging. Also other lactobacilli are identified, but their numbers were found to be significantly lower than *Lb. sakei* and *Lb. curvatus*. *Leuconostoc* and *Weissella* spp. were identified to a lesser extent, underlining possible pitfalls in their identification by traditional methods, or more simply their low presence in the type of Sausages studied. Also in the case of CNC, apart from *S. xylosus* other species were described. *S. carnosus*, *S. simulans*, *S. condimentii*, *S. pulvereri/vitulis*, *S. equorum*, *S. saprophyticus* were identified among the CNC

isolates, and it is interesting to note that some species were identified only when molecular methods were applied. *Kocuria* spp. were identified only in “salame friulano” (Cocolin, et al. 2001b).

It is important to note that the application of molecular methods to characterize strains isolated from different processing plants or fermented Sausages underlined, for both LAB and CNC, a plant or product specificity. This confirms the theory that in a production plant, depending on the process (temperature, humidity and ingredients), there is always a specific selection of microflora that may influence the final characteristics of the products.

5 Final Remarks

Sausage fermentation is a complex series of events in which microorganisms represent key agents for the production of specific compounds and enzymes that allow the transformation of raw meat in a product with new physicochemical and sensory characteristics. In this process the quality of the meat and of the ingredients (salt, spices, nitrate and nitrite), the microorganisms operating the transformations (added as a starter or naturally present) and the fermentation and ripening conditions are fundamental parameters to control in order to obtain final products with the desired organoleptic profile.

It is widely accepted that the contribution of microorganisms, in particular LAB and CNC, is essential to achieve specific sensory characteristics during Sausage fermentations. For this reason, the study of the microflora is an important aspect to take into account. It is worth noting that the microflora of fermented Sausages – independent of the geographic location, ingredients and processing conditions – is always constituted by the same species of LAB and CNC. This aspect is underlining the capability of these species to adapt to the specific environment characterized by a strong selective pressure towards the microorganisms.

Considering the data available on the microflora in fermented Sausages, it can be concluded that there is a good correlation between the results obtained with traditional methods and the ones achieved with molecular methods of identification. With both approaches it has been repeatedly highlighted that species belonging to *Lb. sakei*, *Lb. curvatus* and *S. xylosus* are the best adapted to the Sausage fermentation ecosystem, thereby dominating the microflora present. However, small differences can be observed in cases of traditional products coming from a specific country. As an example, researchers from Greece using traditional methods defined that dry salami was characterized by the presence of *Weissella* members, such as *W. viridescens*, *W. hellenica* and *W. paramensisenteroides* (Samelis, et al. 1994; Papamanoli, et al. 2003). This evidence was not confirmed by molecular methods; in fact, in one study performed using PCR-DGGE followed by 16S rRNA gene sequencing, it was determined that these species were absent in Sausages produced in Greece (Rantsiou, et al. 2005a). The differing results can be explained by simply considering that the studies were performed on different samples, so it is possible

that in the last case Sausages did not contain *Weissella* spp., but it is acceptable to recognize a probable over-estimation of this genera with traditional methods.

With the development of molecular methods of detection and identification new frontiers can be reached. The possibility to profile populations directly in the sample, without the need of traditional isolation and identification, allows scientists to investigate the microbial interrelations *in situ* for the first time. Moreover, recently the genome sequence of *Lb. sakei* 23K, isolated from fermented Sausages, has been published (Chaillou, et al. 2005) and, as a consequence, new information to better understand the role of this microorganism in meat ecosystems can be obtained. As a matter of fact, Hüfner, et al. (2007) used *in vivo* expression technology (IVET) to study the genes of *Lb. sakei* 23K induced during meat fermentation, to determine their role in survival and growth. The results obtained showed that several genes encoded proteins – which are likely to contribute to stress-related functions – were expressed.

Considering the culture-independent methods, once more the capability of *Lb. sakei*, *Lb. curvatus* and *S. xylosus* to dominate the fermentation process is obvious, but new interesting information is also obtained. As a matter of fact, it seems that other species of LAB and CNC are active in the transformation process as well. *Lb. paracasei* was identified by means of the direct PCR-DGGE method in Italian Sausages. Moreover, *L. garvieae* also seems to contribute to the fermentation. In the group of CNC, *S. equorum* and *S. succinus*, although isolated in Sausages at low percentages by traditional methods, were often detected by direct methods, and, therefore, should be considered important.

Future developments in the field of Sausage fermentation can be represented by studies on strain diversity with the final goal of explaining the diversity of available products. Sausages produced in different countries, but also within the same country, are characterized by different organoleptic profiles and sensory characteristics, and this aspect can be only partially explained considering the actual ingredients used. The microflora plays a major role by this point of view, and it is possible that the differences in the sensory characteristics of the products are due to the presence of different strains. This aspect becomes even more interesting considering that in the fermented Sausages studied so far the main species found were the same among the different products. A possible explanation could be the presence within the species of specific strains unique for a specific product. It has been defined, for example, that within the species *Lb. sakei* and *Lb. curvatus* there are populations that can be differentiated based on their provenience (Rantsiou, et al. 2005a). Moreover, a study performed on about 350 LAB strains, isolated from three plants of continental Greece, highlighted the presence of plant-specific populations (Rantsiou, et al. 2006). Little is known about the intraspecies differences of LAB strains involved in the Sausage fermentations. However, the plant-specific clusters found are highlighting that the production conditions (temperature, moisture and ingredients) that differ from plant to plant are possibly able to determine the selection of a specific population, and more precisely of a specific strain within the species, that will take over during transformation, characterizing the final product of the specific plant. This is an impor-

tant aspect to consider in our understanding of the fermentation process, and it needs to be investigated further. Defining and understanding microbial dynamics, as determined by species successions, as well as microbial ecology, as determined by species interactions at each time point and throughout fermentation, is crucial since these are the parameters that will have a great impact on the organoleptic and sensorial characteristics of the final product.

References

- Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K. 1994. Identification of the heme compound co-purified with deoxyribonucleic acid (DNA) from blood stains, a major inhibition of polymerase chain reaction (PCR) amplification. *J. Forensic Sci.* 39, 362–372.
- Ammor, S., Rachman, C., Chaillou, S., Prevost, H., Dousset, X., Zagorec, M., Dufur, E., Chevallier, I. 2005. Phenotypic and genotypic identification of lactic acid bacteria isolated from a small-scale facility producing traditional dry sausages. *Food Microbiol.* 22, 373–382.
- Andrighetto, C., Zampese, L., Lombardi, A. 2001. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). *Lett. Appl. Microbiol.* 33, 26–30.
- American Public Health Association, 1966. Recommended Methods for the Microbiological Examination of Foods. 2nd Ed. APHA Inc., New York.
- Aymerich, T., Martin, B., Garriga, M., Hugas, M. 2003. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. *Appl. Environ. Microbiol.* 69, 4583–4594.
- Aymerich, T., Martin, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S., Hugas, M. 2006. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J. Appl. Microbiol.* 100, 40–49.
- Bacus, J. N. 1986. Fermented meat and poultry products. In: Pearson, A. M. D. (Ed.), *Advances in meat and poultry microbiology*. Macmillan, London, United Kingdom, pp. 123–164.
- Baruzzi, F., Matarante, A., Caputo, L., Morea, M. 2006. Molecular and physiological characterization of natural microbial communities isolated from a traditional Southern Italian processed sausages. *Meat Sci.* 72, 261–269.
- ben Omar, N., Ampe, F. 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl. Environ. Microbiol.* 66, 3664–3673.
- Berthier, F., Ehrlich, S. D. 1998. Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. *FEMS Microbiol. Lett.* 161, 97–106.
- Berthier, F., Ehrlich, S. D. 1999. Genetic diversity within *Lactobacillus sakei* and *Lactobacillus curvatus* and design of PCR primers for its detection using randomly amplified polymorphic DNA. *Int. J. Syst. Bacteriol.* 49, 997–1007.
- Blaiotta, G., Pennacchia, C., Ercolini, D., Moschetti, G., Villani, F. 2003a. Combining denaturing gradient gel electrophoresis of 16S rDNA V3 region and 16S-23S rDNA spacer region polymorphism analyses for the identification of staphylococci from Italian fermented sausages. *Syst. Appl. Microbiol.* 26, 423–433.
- Blaiotta, G., Pennacchia, C., Parente, E., Villani, F. 2003b. Design and evaluation of specific PCR primers for rapid and reliable identification of *Staphylococcus xylosus* strains isolated from dry fermented sausages. *Syst. Appl. Microbiol.* 26, 601–610.
- Blaiotta, G., Pennacchia, C., Villani, F., Ricciardi, A., Tofalo, R., Parente, E. 2004a. Diversity and dynamics of communities of coagulase-negative staphylococci in traditional fermented sausages. *J. Appl. Microbiol.* 97, 271–284.

- Blaiotta, G., Ercolini, D., Mauriello, G., Salzano, G., Villani, F. 2004b. Rapid and reliable identification of *Staphylococcus equorum* by a species-specific PCR assay targeting the *sodA* gene. Syst. Appl. Microbiol. 27, 696–702.
- Blaiotta, G., Casaburi, A., Villani, F. 2005. Identification and differentiation of *Staphylococcus carnosus* and *Staphylococcus simulans* by species-specific PCR assays of *sodA* genes. Syst. Appl. Microbiol. 28, 519–526.
- Buffone, G. J., Demmler, G. J., Schimbor, C. M., Greer, J. 1991. Improved amplification of cytomegalovirus DNA from urine after purification of DNA with glass beads. Clin. Chem. 37, 1945–1949.
- Chaillou, S., Champomier-Vergès, M., Cornet, M., Crutz Le Coq, A.-M., Dudez, A.-M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V., Zagorec, M., 2005. Complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. Nat. Biotechnol. 23, 1527–1533.
- Charteris, W. P., Kelly, P. M., Morelli, L., Collins, J. K. 1997. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int. J. Food Microbiol. 35, 1–27.
- Cocolin, L., Manzano, M., Cantoni, C., Comi, G. 2000. Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausage using a polymerase chain reaction - temperature gradient gel electrophoresis. Lett. Appl. Microbiol. 30, 126–129.
- Cocolin, L., Manzano, M., Cantoni, C., Comi, G. 2001a. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. Appl. Environ. Microbiol. 67, 5113–5121.
- Cocolin, L., Manzano, M., Aggio, D., Cantoni, C., Comi, G. 2001b. A novel polymerase chain reaction (PCR) - denaturing gradient gel electrophoresis (DGGE) for the identification of Micrococcaceae strains involved in meat fermentations. Its application to naturally fermented Italian sausages. Meat Sci. 57, 59–64.
- Cocolin, L., Aggio, D., Manzano, M., Cantoni, C., Comi, G. 2002. An application of PCR-DGGE analysis to profile the yeast populations in raw milk. Int. Dairy J. 12, 407–411.
- Cocolin, L., Mills, D. A. 2003. Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and independent methods. Am. J. Enol. Viticult. 54, 125–130.
- Cocolin, L., Rantsiou, K., Iacumin, L., Urso, R., Cantoni, C., Comi, G. 2004. Study of the ecology of fresh sausages and characterization of populations of lactic acid bacteria by molecular methods. Appl. Environ. Microbiol. 70, 1883–1894.
- Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C., Comi, G. 2006a. Multiphasic approach to study the bacterial ecology of fermented sausages inoculated with a commercial starter cultures. Appl. Environ. Microbiol. 72, 942–945.
- Cocolin, L., Urso, R., Rantsiou, K., Cantoni, C., Comi, G. 2006b. Dynamics and characterization of yeasts during natural fermentation of Italian sausages. FEMS Yeast Res. 6, 692–701.
- Collins, M. D., Rodriguez, U., Ash, C., Aguirre, M., Farrow, J. E., Martinez-Murcia, A., Philips, B. A., Williams, A. M., Wallbanks, S. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse-transcriptase sequencing of 16S ribosomal-RNA. FEMS Microbiol. Lett. 77, 5–12.
- Comi, G., Urso, R., Iacumin, L., Rantsiou, K., Cattaneo, P., Cantoni, C., Cocolin, L. 2005. Characterization of naturally fermented sausages produced in the North East of Italy. Meat Sci. 69, 381–392.
- Coppola, S., Mauriello, G., Aponte, M., Moschetti, G., Villani, F. 2000. Microbial succession during ripening of Naples-type salami, a southern Italian fermented sausage. Meat Sci. 56, 321–329.
- Corbiere Morot-Bizot, S., Talon, R., Leroy, S. 2003. Development of specific primers for a rapid and accurate identification of *Staphylococcus xylosus*, a species used in food fermentation. J. Microbiol. Meth. 55, 279–286.

- Corbiere Morot-Bizot, S., Talon, R., Leroy, S., 2004. Development of a multiplex PCR for the identification of *Staphylococcus* genus and four staphylococcal species isolated from food. *J. Appl. Microbiol.* 97, 1087–1094.
- Corbiere Morot-Bizot, S., Leroy, S., Talon, R., 2006. Staphylococcal community of a small unit manufacturing traditional dry fermented sausages. *Int. J. Food Microbiol.* 108, 210–217.
- Dahllof, I., Baillie, H., Kjelleberg, S., 2000. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecific heterogeneity. *Appl. Environ. Microbiol.* 66, 3376–3380.
- de Man, J. C., Rogosa, M., Sharpe, M. E., 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* 23, 130–138.
- Demeyer, D. I., 1982. Stoichiometry of dry sausage fermentation. *Antonie Leeuwenhoek* 48, 414–416.
- Demeyer, D. I., Verplaetse, A., Gistelink, M., 1986. Fermentation of meat: an integrated process. *Belg. J. Food Chem. Biotechnol.* 41, 131–140.
- Di Maria, S., Basso, A. L., Santoro, E., Grazia, L., Coppola, R., 2002. Monitoring of *Staphylococcus xylosus* DSM 20266 added as starter during fermentation and ripening of soppressata molisana, a typical Italian sausage. *J. Appl. Microbiol.* 92, 158–164.
- Drosinos, E. H., Mataragas, M., Xiraphi, N., Moschonas, G., Gaitis, F., Metaxopoulos, J., 2005. Characterization of the microbial flora from traditional Greek fermented sausages. *Meat Sci.* 69, 307–317.
- Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. Behavior of variable V3 region from 16S rDNA of important lactic acid bacteria in denaturing gradient gel electrophoresis. *Curr. Microbiol.* 42, 199–202.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Methods* 56, 297–314.
- Fadda, S., Vignolo, G., Holgado, A. P. R., Oliver, G., 1998. Proteolytic activity of *Lactobacillus* strains isolated from dry-fermented sausages on muscle sarcoplasmic proteins. *Meat Sci.* 49, 11–18.
- Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrà, F., 1999a. Characterization of muscle sarcoplasmic and myofibrillar protein hydrolysis caused by *Lactobacillus plantarum*. *Appl. Environ. Microbiol.* 65, 3540–3546.
- Fadda, S., Sanz, Y., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrà, F., 1999b. Hydrolysis of pork muscle sarcoplasmic proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. *Appl. Environ. Microbiol.* 65, 578–584.
- Fadda, S., Oliver, G., Vignolo, G., 2002. Protein degradation by *Lactobacillus plantarum* and *Lactobacillus casei* in a sausage model system. *J. Food Sci.* 67, 1179–1183.
- Fontana, C., Vignolo, G., Cocconcelli, P. S., 2005a. PCR-DGGE analysis for the identification of microbial populations from Argentinean dry fermented sausages. *J. Microbiol. Meth.* 63, 254–263.
- Fontana, C., Cocconcelli, P. S., Vignolo, G., 2005b. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *Int. J. Food Microbiol.* 103, 131–142.
- Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* 205, 31–36.
- Greco, M., Mazzette, R., De Santis, E. P. L., Corona, A., Cosseddu, A. M., 2005. Evolution and identification of lactic acid bacteria isolated during the ripening of Sardinian sausages. *Meat Sci.* 69, 733–739.
- Gürtler, V., Stanisich, V. A., 1996. New approaches to typing and identification of bacteria using the 16S-23S rRNA spacer region. *Microbiol.* 141, 1255–1265.
- Hammes, W. P., Bantleon, A., Min, S., 1990. Lactic acid bacteria in meat fermentation. *FEMS Microbiol. Rev.* 87, 165–174.
- Hammes, W. P., Knauf, H. J., 1994. Starters in processing of meat products. *Meat Sci.* 36, 155–168.
- Head, I. M., Saunders, J. R., Pickup, R. W., 1998. Microbial evolution, diversity and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35, 1–21.

- Hertel, C., Ludwig, W., Obst, M., Vogel, R. F., Hammes, W. P., Schleifer, K. H. 1991. 23S rRNA-targeted oligonucleotide probes for the rapid identification of meat lactobacilli. *Syst. Appl. Microbiol.* 14, 173–177.
- Heuer, H., Smalla, K. 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gel electrophoresis (TGGE) for studying soil microbial communities. In van Elsas, J. D., Wellington, E. M. H. and Trevors, J. T. E. (Eds), *Modern soil microbiology*. Marcel Dekken Inc., New York, pp. 353–373.
- Hüfner, E., Markieton, T., Chaillou, S., Crutz-Le Coq, A.M., Zagorec, M., Hertel, C. 2007. Identification of *Lactobacillus sakei* genes induced during meat fermentation and their role in survival and growth. *Appl. Environ. Microbiol.* 73, 2522–2531.
- Hugas, M., Garriga, M., Aymerich, M.T. 2003. Functionality of enterococci in meat products. *Int. J. Food Microbiol.*, 88, 223–233.
- Hugenholtz, P., Goebel, B. M., Pace, N. R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* 180, 4765–4774.
- Hugenholtz, P., Pace, N. R. 1996. Identifying microbial diversity in the natural environment: a molecular phylogenetic approach. *Trends Biotechnol.* 14, 90–97.
- Iacumin, L., Comi, G., Cantoni, C., Cocolin, L. 2006a. Molecular and technological characterization of *Staphylococcus xylosus* isolated from Italian naturally fermented sausages by RAPD, Rep-PCR and Sau-PCR analysis. *Meat Sci.* 74, 281–288.
- Iacumin, L., Comi, G., Cantoni, C., Cocolin, L. 2006b. Ecology and dynamics of coagulase-negative cocci isolated from naturally fermented Italian sausages. *Syst. Appl. Microbiol.* 29, 480–486.
- Janse, I., Bok, J., Zwart, G. 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J. Microbiol. Meth.* 57, 279–281.
- Lee, J., Jang, J., Kim, B., Kim, J., Jeong, G., Han, H. 2004. Identification of *Lactobacillus sakei* and *Lactobacillus curvatus* by multiplex PCR-based restriction enzyme analysis. *J. Microbiol. Meth.* 59, 1–6.
- Lerche, M., Reuter, G. 1960. A contribution to the method of isolation and differentiation of aerobic “lactobacilli” (Genus *Lactobacillus* Beijerinck). *Arch. Hyg. Bakteriologie* 179, 354–370.
- Lücke, F. K. 1974. Fermented sausages. In: Wood, B. J. B. (Ed.), *Microbiology of fermented foods*. Applied Science Publishers, London, England, pp. 41–49.
- Lücke, F. K. 2000. Utilization of microbes to process and preserve meat. *Meat Sci.* 56, 105–115.
- Martin, B., Jofré, A., Garriga, M., Pla, M., Aymerich, T. 2006a. Rapid quantitative detection of *Lactobacillus sakei* in meat and fermented sausages by real-time PCR. *Appl. Environ. Microbiol.* 72, 6040–6048.
- Martin, B., Garriga, M., Hugas, M., Bover-Cid, S., Veciana-Nogues, M. T., Aymerich, T. 2006b. Molecular, technological and safety characterization of Gram-positive catalase-positive cocci from slightly fermented sausages. *Int. J. Food Microbiol.* 107, 148–158.
- Mauriello, G., Casaburi, A., Blaiotta, G., Villani, F. 2004. Isolation and technological properties of coagulase negative staphylococci from fermented sausages of Southern Italy. *Meat Sci.* 67, 149–158.
- Metaxopoulos, J., Samelis, J., Papadelli, M. 2001. Technological and Microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry fermented sausage in Greece. *Ital. J. Food Sci.* 13, 3–18.
- Mills, D. A., Johannsen, E. A., Cocolin, L. 2002. Yeast diversity and persistence in botrytis-affected wine fermentation. *Appl. Environ. Microbiol.* 68, 4884–4893.
- Molly, K., Demeyer, D., Johansson, G., Raemaekers, M., Ghistelinek, M., Geenen, I. 1997. The importance of meat enzymes in ripening and flavor generation in dry fermented sausages. First results of a European project. *Food Chem.* 59, 539–545.
- Muyzer, G., Hottentrager, S., Teske, A., Wawer, C. 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA - a new molecular approach to analyse the genetic diversity of mixed microbial communities. In: Akkermans, A. D. L., van Elsas, J. D. and de Bruijn, F. J. E. (Eds), *Molecular Microbial Ecology Manual*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1–23.

- Myers, R. M., Maniatis, T., Lerman, L. S. 1987. Detection and localization of single base change by denaturing gel electrophoresis. *Meth. Enzymol.* 155, 501–527.
- Nissen, H., Dainty, R. 1995. Comparison of the use of rRNA probes and conventional methods in identifying strains of *Lactobacillus sake* and *L. curvatus* isolated from meat. *Int. J. Food Microbiol.* 25, 311–315.
- Nour, M. 1998. 16S-23S and 23S-5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. *Res. Microbiol.* 149, 433–448.
- Papamanoli, E., Kotzekidou, P., Tzanetakis, N., Litopoulou-Tzanetaki, E. 2002. Characterization of *Micrococcaceae* isolated from dry fermented sausages. *Food Microbiol.* 19, 441–449.
- Papamanoli, E., Tzanetakis, N., Lipopoulou-Tzanetaki, E., Kotzekidou, P. 2003. Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties. *Meat Sci.* 65, 859–867.
- Pereira, C. I., Barreto Crespo, M. T., San Romao, M. V. 2001. Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii*. *Int. J. Food Microbiol.* 68, 211–216.
- Powell, H. A., Gooding, C. M., Garreit, S. D., Lund, B. M., McKee, R. A. 1994. Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. *Lett. Appl. Microbiol.* 18, 59–61.
- Rantsiou, K., Comi, G., Cocolin, L. 2004. The *rpoB* gene as a target for PCR-DGGE analysis to follow lactic acid bacteria population dynamics during food fermentations. *Food Microbiol.* 21, 481–487.
- Rantsiou, K., Drosinos, E. H., Gialitaki, M., Urso, R., Krommer, J., Gasparik-Reichardt, J., Toth, S., Metaxopoulos, I., Comi, G., Cocolin, L. 2005a. Molecular characterization of *Lactobacillus* species isolated from naturally fermented sausages produced in Greece, Hungary and Italy. *Food Microbiol.* 22, 19–28.
- Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L. 2005b. Culture dependent and independent methods to investigate the microbial ecology of Italian fermented sausages. *Appl. Environ. Microbiol.* 71, 1977–1986.
- Rantsiou, K., Iacumin, L., Cantoni, C., Comi, G., Cocolin, L. 2005c. Ecology and characterization by molecular methods of *Staphylococcus* species isolated from fresh sausages. *Int. J. Food Microbiol.* 97, 277–284.
- Rantsiou, K., Cocolin, L. 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. *Int. J. Food Microbiol.* 108, 255–267.
- Rantsiou, K., Drosinos, E. H., Gialitaki, M., Metaxopoulos, I., Comi, G., Cocolin, L. 2006. Use of molecular tools to characterize *Lactobacillus* spp. isolated from Greek traditional fermented sausages. *Int. J. Food Microbiol.* 112, 215–222.
- Rebecchi, A., Crivori, S., Sarra, P. G., Coconcelli, P. S. 1998. Physiological and molecular techniques for the study of bacterial community development in sausage fermentation. *J. Appl. Microbiol.* 84, 1043–1049.
- Reuter, G. 1972. Experimental ripening of dry sausages using lactobacilli and micrococci starter cultures. *Fleischwirtschaft* 52, 465–468, 471–473.
- Rossen, L., Nørskov, P., Holmström, K., Rasmussen, O. F. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *Int. J. Food Microbiol.* 17, 37–45.
- Rossi, F., Tofalo, R., Torriani, S., Suzzi, G. 2001. Identification by 16S-23S rDNA intergenic region amplification, genotypic and phenotypic clustering of *Staphylococcus xylosus* strains from dry sausages. *J. Appl. Microbiol.* 90, 365–371.
- Samelis, J., Aggelis, G., Metaxopoulos, J. 1993. Lipolytic and microbial changes during the natural fermentation and ripening of Greek dry sausages. *Meat Sci.* 35, 371–385.
- Samelis, J., Maurogenakis, F., Metaxopoulos, J. 1994. Characterization of lactic acid bacteria isolated from naturally fermented Greek dry salami. *Int. J. Food Microbiol.* 23, 179–196.

- Samelis, J., Tsakalidou, E., Metaxopoulos, J., Kalantzopoulos, G. 1995. Differentiation of *Lactobacillus sake* and *Lact. curvatus* isolated from naturally fermented Greek dry salami by SDS-PAGE of whole cell proteins. *J. Appl. Bacteriol.* 78, 157–163.
- Samelis, J., Metaxopoulos, J., Vlasi, M., Pappa, A. 1998. Stability and safety of traditional Greek salami - a microbiological ecology study. *Int. J. Food Microbiol.* 23, 179–196.
- Santos, N. N., Santos-Mendosa, R. C., Sanz, Y., Bolumar, T., Aristoy, M.-C., Toldrà, F. 2001. Hydrolysis of pork muscle sarcoplasmatic proteins by *Debaryomyces hansenii*. *Int. J. Food Microbiol.* 68, 199–206.
- Sanz, B., Selgas, D., Parejo, I., Ordonez, J. A. 1988. Characteristics of lactobacilli isolated from dry fermented sausages. *Int. J. Food Microbiol.* 6, 199–205.
- Sanz, Y., Hernandez, M., Ferrus, M. A., Hernandez, J. 1998. Characterization of *Lactobacillus sake* isolates from dry-cured sausage by restriction fragment length polymorphism analysis of the 16S rRNA gene. *J. Appl. Microbiol.* 84, 600–606.
- Sanz, Y., Fadda, S., Vignolo, G., Aristoy, M.-C., Oliver, G., Toldrà, F. 1999. Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. *Int. J. Food Microbiol.* 53, 115–125.
- Schleifer, K. H. 1986. Gram positive cocci. In: Sneath, P. H. A., Mair, N. S. and Holt, J. G. (Eds), *Bergey's Manual of Systematic Bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md., pp. 999–1003.
- Talon, R., Walter, D., Chartier, S., Barriere, C., Montel, M. C. 1999. Effect of nitrate and incubation conditions on the production of catalase and nitrate reductase by staphylococci. *Int. J. Food Microbiol.* 52, 47–56.
- Urso, R., Comi, G., Cocolin, L. 2006. Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. *Syst. Appl. Microbiol.* 29, 671–680.
- van der Gucht, K., Sabbe, K., de Meester, L., Vloemans, N., Zwart, G., Gillis, M., Vyverman, W. 2001. Contrasting bacterioplankton community composition and seasonal dynamics in two neighboring hypertrophic freshwater lakes. *Environ. Microbiol.* 3, 680–690.
- Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51, 221–271.
- Yost, C. K., Nattress, F. M. 2000. The use of multiplex PCR reactions to characterize populations of lactic acid bacteria associated with meat spoilage. *Lett. Appl. Microbiol.* 31, 129–133.
- Yu, Z., Morrison, M. 2004. Comparison of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70, 4800–4806.
- Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., Reid, G. 1998. Differentiation of *Lactobacillus* species by molecular typing. *Appl. Environ. Microbiol.* 64, 2418–2423.

Chapter 4

Sourdough Fermentations

Rudi F. Vogel and Matthias A. Ehrmann

Abstract The fermentative biota of the majority of different sourdough types consists of lactic acid bacteria (LAB), most of which belong to the genus *Lactobacillus*, and yeasts. The exploitation of these organisms emerging from a long history in sourdough fermentations forms an emerging field for baking applications and design of added-value food. The deliberate use of functional traits within these bacteria is strongly supported by knowledge of their diversity, phylogenetic and environmental status, the characterization of their genome structure, gene regulation and metabolic potential. This knowledge base is required to achieve a status of qualified presumption of safety (QPS) as recently proposed by the European Authority for Food Safety (EFSA). Molecular methodology in taxonomy and genetics are the major contributors to gain these insights in biodiversity, behavior and functionality of sourdough microorganisms.

1 Introduction

In principle, sourdough is a fermented mixture of flour and water. However, different raw materials, country-specific artisanal propagation procedures, consumer demands and a very long tradition have led to an unmanageable variety of sourdoughs all over the world.

Based on differences in production technology sourdoughs have been roughly classified into three types (Böcker, et al. 1995; De Vuyst and Neysens 2005). Type I sourdoughs are characterized by continuous refreshments keeping the microorganisms in a highly active state. Within Europe these traditional fermentations usually take place at ambient temperature (max. 30 °C) and consist of different fermentation steps (e.g., fresh sour, basic sour and full sour) which are usually found in traditional rye sourdough production. Type II sourdoughs are large scale one-step fermentations of semi-fluid silo preparations. These processes last for up to five days at increased temperatures (max. 50 °C), and the acid content at pH of < 3.5 is much more than in type I sourdoughs. Dried sourdough products that are used as acidifiers and/or aroma carriers for bread making are summarized under type III sourdoughs. It is quite obvious that each type of fermentation harbors its own microflora.

Due to the nature of the substrate, and in contrast to other food fermentations, all sourdough fermentations are run under non-aseptic conditions. Nevertheless, depending on fermentation regimes (time/temperature) and choice of flour (wheat, rye, a mixture thereof or others) each sourdough may be regarded as a specific ecosystem whose microbial consortia are highly stable in cell numbers, diversity and strain composition for long periods of time. Sourdoughs are numerically dominated by a variety of specifically adapted lactic acid bacteria (LAB) often occurring at numbers $> 10^8$ colony forming units/g, which may be in co-existence or possibly in symbiosis with typical yeasts whose numbers are orders of magnitude lower (Gobbetti 1998; Vogel, et al. 2002).

Efforts have been made to isolate and describe the sourdough microbiota in their phylogenetic position, ecological status and functional properties revealing that the understanding of their competitiveness, adaptation, metabolism and stress response requires their genetic analysis and modification. The most obvious contribution of the microflora is souring by secretion of lactic acid and acetic acid. Amongst others, functional traits like secretion of aroma compounds or aroma precursors, polysaccharide production and antifungal activity are recognized as important factors.

Recent developments indicate that sourdough LAB have an underestimated potential for many applications in bread making and design of added-value food. Exploitation of traditionally known and upcoming new species from this environment, characterization of their genome structure and flexibility, their gene regulation or metabolic potential as a result of gene presence or expression are examples which delineate sourdough LAB as an emerging field. Molecular taxonomy, genetics and proteomics are major contributors to these developments.

2 History of LAB Taxonomy

The taxonomy of LAB occurring in sourdough is strictly linked to the taxonomy of gram positive low GC bacteria, which is fluxionary and has been the subject of countless studies. This review focuses on LAB occurring in sourdough fermentations wherefore the more broadly interested reader may be referred to excellent reviews dealing with LAB as a whole written by Axelsson (1998), Stiles and Holzapfel (1997) or Schleifer and Ludwig (1995a, b).

Sourdough LAB may be considered a subset of LAB and some are known since the term LAB became manifested as the circumscription of a group of gram positive, non-sporing and strictly fermentative organisms with lactic acid as the major metabolic end product.

The majority of bacterial species regularly isolated from sourdough, or used as sourdough starter, fall (with only few exceptions) into one of the four genera: *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella*. A survey of LAB that have been isolated from sourdough is shown in Table 4.1. The highest number of different species primarily isolated from sourdoughs is found in *Lactobacillus*. The entire physiological and genetic heterogeneity of this genus is echoed in sourdough LAB.

Table 4.1 Survey of Lactic Acid Bacteria Isolated from Sourdoughs

Country	Product	Lactic Acid Bacteria	References
Germany	Wheat bread	<i>Lb. delbrueckii</i> , " <i>Lb. Leichmannii</i> ," <i>Lb. plantarum</i> , <i>Lb. casei</i> , <i>Lb. fermentum</i> , <i>L. pasteurianus</i> , <i>Lb. buchneri</i> , <i>Lb. brevis</i>	Spicher 1959
USA	San Francisco bread	<i>Lb. sanfranciscensis</i>	Kline and Sugihara 1971
Italy	Panettone	<i>Lb. brevis</i> , <i>Lb. plantarum</i>	Galli and Ottogalli 1973
	n.d.	<i>Lb. plantarum</i> , <i>Lb. brevis</i> , <i>Lc. mesenteroides</i> , <i>P. cerevisiae</i>	Azar, et al. 1977
Germany	Rye bread	<i>Lb. acidophilus</i> , <i>Lb. farciminis</i> , <i>Lb. alimentarius</i> , <i>Lb. casei</i> , <i>Lb. plantarum</i> , <i>Lb. brevis</i> , <i>Lb. sanfranciscensis</i> , <i>Lb. fructivorans</i> , <i>Lb. fermentum</i> , <i>Lb. buchneri</i>	Spicher and Schröder 1978
Germany	Wheat bread starter	<i>Lb. brevis</i> , <i>Lb. plantarum</i> , <i>Lb. acidophilus</i> , <i>Lb. casei</i> , <i>Lb. plantarum</i> , <i>Lb. farciminis</i> , <i>Lb. alimentarius</i> , <i>Lb. brevis</i> , <i>Lb. buchneri</i> , <i>Lb. fermentum</i> , <i>Lb. fructivorans</i> , <i>Lb. brevis var. linderi</i>	Barber, et.al. 1983 Spicher 1984
Germany	Panettone, Wheat bread	Pediococci <i>Lb. casei</i> , <i>Lb. plantarum</i> , <i>Lb. farciminis</i> , <i>Lb. alimentarius</i> , <i>Lb. brevis</i> , <i>Lb. buchneri</i> , <i>Lb. fermentum</i> , <i>Lb. fructivorans</i> , <i>Lb. sanfranciscensis</i> , <i>Lb. hilgardii</i> , " <i>Lb. homohiochii</i> " <i>Lc. viridescens</i>	Spicher 1987
Italy	Panettone, Brioches, Wheat bread, Rye bread, Crackers	<i>Lb. sanfranciscensis</i> , <i>Lb. fermentum</i> , <i>Lb. plantarum</i> , <i>Lc. mesenteroides</i> , <i>Pediococcus</i>	Galli, et al. 1988
Spain	Wheat bread	<i>Lb. plantarum</i> , <i>Lb. brevis</i> , <i>Lc. mesenteroides</i> , <i>E. faecium</i>	Barber and Baguena 1988
France	Wheat bread	<i>Lc. mesenteroides</i> , <i>Lb. acidophilus</i> , <i>Lb. brevis</i> , <i>Lb. delbrueckii</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>P. pentosaceus</i>	Infantes and Tourner 1991
Italy	Wheat bread	<i>Lb. sanfranciscensis</i> , <i>Lb. plantarum</i> , <i>Lb. farciminis</i>	Gobbetti, et al. 1991, 1994
Italy	Wheat bread, Pandoro, Panettone, Focaccia	<i>Lb. plantarum</i> , <i>Lb. brevis</i> , <i>Lb. fermentum</i>	Sarra, et al. 1992
Italy	Wheat bread	<i>Lb. fermentum</i> , <i>Lb. brevis</i>	Foschino, et al.1995

(continued)

Table 4.1 Survey of Lactic Acid Bacteria Isolated from Sourdoughs (continued)

Country	Product	Lactic Acid Bacteria	References
Germany	Rye bread	<i>Lb. pontis</i>	Vogel, et al. 1994
Germany	Rye bread	<i>Lb. panis</i>	Wiese, et al. 1996
Sudan	Kisra, Sorghum bread	<i>Lb. reuteri</i> , <i>Lb. pontis-like</i> , <i>Lb. fermentum</i> , <i>Lb. amylovorus</i>	Hamad, et al. 1997
Japan	n.d.	<i>L. paralimentarius</i>	Cai, et al. 1999
Germany	Type II Sourdough	<i>L. frumenti</i>	Müller, et al. 2000
Germany	Type II Sourdough	<i>Lb. amylovorus</i> , <i>Lb. frumenti</i> , <i>Lb. pontis</i> , <i>Lb. reuteri</i>	Müller, et al. 2001
Italy	Wheat bread	<i>Lb. sanfranciscensis</i> , <i>Lb. alimentarius</i> , <i>Lb. brevis</i> , <i>Lb. plantarum</i> , <i>Lb. fermentum</i> , <i>Lb. acidophilus</i> , <i>Lb. delbrueckii</i> , <i>Lc. citreum</i> , <i>L. lactis</i> , <i>W. confusa</i>	Corsetti, et. al. 2001
Greece	Wheat bread	<i>Lb. sanfranciscensis</i> , <i>Lb. brevis</i> , <i>Lb. brevis-like</i> , <i>Lb. paralimentarius</i> , <i>W. cibaria</i>	De Vyust, et al. 2002
Germany	Wheat, Rye bread	<i>Lb. mindensis</i> , <i>Lb. sanfranciscensis</i>	Ehrmann, et al. 2003
Germany	Type I	<i>Lb. sanfranciscensis</i> , <i>Lb. Mindensis</i>	Meroth, et al. 2003
	Type II	<i>Lb. crispatus</i> , <i>Lb. pontis</i> , <i>Lb. panis</i> , <i>Lb. frumenti</i>	
Italy	Wheat sourdough	<i>Lb. rossiae</i>	Corsetti, et al. 2005
Belgium		<i>Lb. acidifarinae</i>	Vancanneyt, et al. 2005
France		<i>Lb. nantensis</i>	Valcheva, et al. 2006
Belgium		<i>Lb. zymae</i>	Vancanneyt, et al. 2005
South Korea	wheat sourdough	<i>Lb. siliginis</i>	Aslam, et al. 2006
Germany	Type II	<i>Lb. secaliphilus</i>	Ehrmann, et al. 2007
Belgium	sourdough mixture of wheat, rye and spelt flour	<i>Lb. namurensis</i>	Scheirlinck, et al. 2007
Germany	wheat	<i>Lb. hammesii</i>	Valcheva, et al. 2005

There exists no discernible emphasis on any of the fermentation types classically dividing lactobacilli in obligately homofermentative, facultatively heterofermentative and obligately heterofermentative strains. Also, their G + C content spans 34 percent to 54 percent and, thus, has little power to discriminate sourdough LAB. Therefore, a look into sourdough LAB taxonomy must begin with a short description of general LAB taxonomy.

Various revisions and improvements have been made including additional physiological and chemotaxonomical data such as major fermentation pathways, carbohydrate patterns, configuration of lactic acid produced or analysis of peptidoglycan type. But due to their phenotypic nature their taxonomic usefulness is limited by a loss of discrimination and significance of closely related organisms. The uncertainties raised by the variability and instability of certain phenotypic characters and the dependence of culturing conditions are diminished by using molecular (DNA-based) characteristics in taxonomy including genotypic tools such as DNA-DNA

hybridizations, comparative sequencing and the use of gene probes in hybridization or PCR assays. Their operational capacity, the reliability and discriminatory power led to an exponentially increasing number of newly described species during the last decade, e. g. *Lb. pontis* (Vogel, et al. 1994); *Lb. panis* (Wiese, et al. 1996); *Lb. paralimentarius* (Cai, et al. 1999); *Lb. frumenti* (Müller, et al. 2000a); *Lb. mindensis* (Ehrmann, et al. 2003); *Lb. zymae*; *Lb. acidifarinae* (Vancanneyt, et al. 2005); *Lb. spicheri* (Meroth, et al. 2004); *Lb. hammesii* (Valcheva, et al. 2005); *Lb. rossiae* (Corsetti, et al. 2005); *Lb. silliginis* (Aslan, et al. 2006); *Lb. namurensis* (Scheirlinck, et al. 2007), and *Lb. secaliphilus* (Ehrmann, et al. 2007).

Nevertheless, both, phenotypic and genotypic characterization should be used as equal parts of a polyphasic approach for classification purposes. But, whenever reliable and fast identification is in demand, DNA-based techniques are advantageous.

3 Phylogeny and Diversity of Sourdough LAB

The phylogenetic position of taxa is based on the comparison of marker genes that are characterized by their ubiquitous distribution, their high degree in sequence conservation, and their functional equivalence (Ludwig, et al. 1998). Genes encoding ribosomal RNA – both 16S and 23S rRNA – comprising conserved and variable domains, are chosen for most phylogenetic studies and act as the gold standard in bacterial systematics. All genera added on LAB are part of the so-called *Clostridium* branch, which is characterized by a relatively low G + C content (<55 mol %) of the genome. The phylogenetic situation of LAB as it is currently presented is based on 16S rRNA genes and shows a clear separation of the genera. A phylogenetic tree based on 16S rRNA gene sequences comparisons of sourdough LAB is shown in Fig. 4.1.

Six species of *Pediococcus* are intermixed with more than 50 homo- and heterofermentative *Lactobacillus* spp. in the so-called *Lb. casei*- and *Pediococcus*-group (Collins, et al. 1991; Schleifer and Ludwig 1995a, b).

Two additional phylogenetic groups are formed by strict homofermenters in the so called *Lb. delbrueckii*-group and the exclusively heterofermenting *Lb. reuteri*-group. Sourdough LAB are dispread over all three lactobacilli subgroups (Fig. 4.1). Sourdough LAB appear to be restricted to the LAB core genera *Lactobacillus*, *Leuconostoc* and *Weissella*. No correlation of the phylogenetic position is found with fermentation type, physiology or hitherto established biochemical markers. One exception is the clear cut separation of strict homofermenters. A weak ecological correlation may be visible in the *Lb. reuteri*-group which exclusively consists of strains either isolated from human/animal or cereal fermentation sources.

The ability to discriminate species using 16S rRNA genes is different in diverse genera. Resolving power of 16S rRNA is sometimes insufficient to guarantee correct delineation of bacterial species. This becomes evident as more and more closely related strains with sequence similarities nearby or above the actually accepted boundary of 97 percent (Stackebrandt and Goebel 1994) are found. Nevertheless, these isolates are often diagnosable by discriminative phenotypic properties and merit separate species status, if an overall genomic similarity below

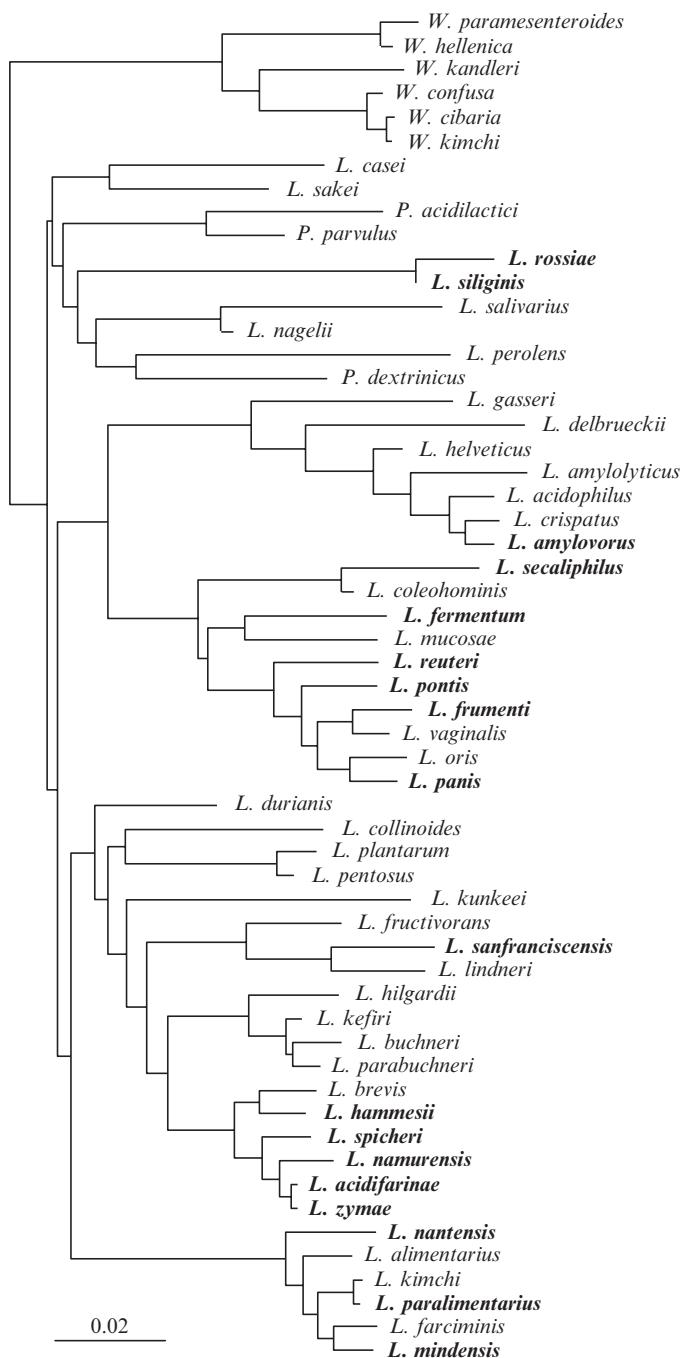


Fig. 4.1 Dendrogram showing the phylogenetic position of sourdough lactobacilli based on 16S rDNA sequence comparison. The tree was constructed using the neighbor-joining method. Bar, 0.02 nucleotide substitutions per 100. Species primarily isolated from sourdough are indicated in bold

70 percent can be demonstrated (Wayne, et al. 1987). Importantly, due to the complex correlation between 16S rRNA sequence similarity and DNA-DNA hybridization similarity, the latter is the more important criterion for lineation of genospecies in closely related organisms (Keswani and Whitman 2001).

Multi-locus sequence analysis (MLSA) becomes a feasible way to get additional phylogenetic information to circumvent the problem of resolving power of single markers (Urwin and Maiden 2003, Palys, et al. 1997; Ventura, et al. 2006). Recently, enzyme-encoding genes have been used for phylogenetic analysis in LAB. Useful candidate genes that have been proven to allow reliable identification and phylogenetic analyses, particularly with regard to sourdough strains, are the protein encoding genes [e.g., phenylalanyl-tRNA synthetase alpha-subunit (*phe*), DNA-dependent RNA polymerase alpha-subunit (*rpoA*) (Naser, et al. 2005; De Vuyst and Vancanneyt 2007), recombinase A (*recA*) (Felis, et al. 2001), 6-phosphogluconate dehydrogenase and elongation factor (*tuf*) (Ehrmann and Vogel 2005)].

The malolactic enzyme separated *Pediococci* and *Lactobacillus* species, otherwise intermixed in 16S rRNA gene-based analyses (Grosillier and Lonvaud-Funel 1999).

4 Diversity of Sourdough Yeasts

In addition to LAB, yeasts contribute to dough leavening (Sugihara, et al. 1971), bread flavor (Martinez, et al. 1990a; 1990b; Hansen and Hansen 1994; Damiani, et al. 1996; Corsetti, et al. 1998) and influence the dough stickiness and rheology as well as crust color, crumb texture, and firmness of the bread (Antuna, et al. 1993; Collar, et al. 1998). As these activities are species- or even strain-specific, a special interest arose to elucidate occurrence and dominance of the sourdough yeasts to control their composition by adjusting the fermentation conditions to the ecological requirements. The diversity of yeast isolated from sourdoughs is as diverse as different types of dough exist. The variability in number and species found depends on several technological factors (e.g., fermentation time, temperature, degree of hydration) as well as type of cereals used. The most frequent yeast species detected in sourdoughs are *Saccharomyces cerevisiae*, *Candida krusei*, *C. milleri*, *Pichia anomala*, *Pi. subpelliculosa*, *S. exiguus*, *Torulaspora holmi* and *C. humilis* (Sugihara, et al. 1971; Galli and Ottogalli 1973; Spicher and Schröder 1980; Barber, et al. 1983; Salovaara and Savolainen 1984; Suihko and Mäkinen 1984; Spicher 1987; Boraam, et al. 1993; Gobetti, et al. 1994b; Iorizzo, et al. 1995; Rossi 1996; Foschino and Galli 1997; Pulvirenti, et al. 2001; Gullo, et al. 2002).

5 Culture-independent Methods to Analyze Sourdough LAB

One of the most important features of a molecular approach is the possibility to monitor the presence, succession and persistence of microorganisms in the ecosystem without any cultivation. The estimation of microbial profiles in foods is often incorrectly

estimated with regard to identity, number and composition of bacteria. A comprehensive review about culture-independent strategies for evaluating microbial communities in food-associated ecosystems was written by Giraffa and Neviani (2001). Denaturing or temperature gradient gel electrophoresis (DGGE or TGGE, respectively) of rDNA amplicons (PCR-DGGE) is well established in evaluation of compositions and activity over time in complex ecosystems (Myuzer and Smalla 1998; Heilig, et al. 2002). Species-specific rDNA amplicons are electrophoretically separated according to differences in sequences. Ampe, et al. (2001) investigated the bacterial flora during the spontaneous non-grain fermentation of sour cassava starch and reported the presence of *Lb. plantarum* and close relatives to *Lb. pontis* and *Lb. panis* (Ampe, et al. 2002), among others. In a second study he found *Lb. plantarum*, *Lb. delbrueckii* and *Lb. fermentum* in fermented maize foods (Ampe and Miambi 2000). LAB-specific DGGE were used to monitor changes in the bacterial population during different sourdough fermentations that have been performed under different ecological conditions (Meroth, et al. 2003; Gatto and Torriani 2004; Settanni, et al. 2006).

Meroth, et al. characterized the dynamics of the microbiota in four different sourdoughs produced with a starter mixture of three commercially available sourdough starters and a baker's yeast containing various species of LAB. In sourdough A (traditional process with rye flour), *Lb. sanfranciscensis* and *Lb. mindensis*, were detected. In rye flour sourdoughs B and C, which differed in the process temperature, *Lb. crispatus* and *Lb. pontis* exclusively became the predominant species in sourdough B (30 °C), and *Lb. crispatus*, *Lb. panis*, and *Lb. frumenti* became the predominant species in sourdough C (40 °C). On the other hand, in sourdough D (corresponding to sourdough C, but produced with rye bran), *Lb. johnsonii* and *Lb. reuteri* were found. The results of PCR-DGGE were consistent with those obtained by culturing, except for sourdough B, in which *Lb. fermentum* was also detected. Isolates of the species *Lb. sanfranciscensis* and *Lb. fermentum* were shown by randomly amplified polymorphic DNA (RAPD)-PCR analysis to originate from the commercial starters and the baker's yeast, respectively.

A multiplex PCR/PCR-DGGE combined system was developed by Settanni, et al. (2006). Total DNA from dough was first subjected to grouping multiplex PCR, and then the bands characteristic of the dough were re-amplified and separated by DGGE. The system was applied to analyze four artisan wheat sourdoughs (A to D) collected in the Abruzzo region (central Italy) at the end of fermentation (6 hours at about 25 °C). Analysis showed a prevalence of *Lb. plantarum* and *Lb. sanfranciscensis* – common species in Italian sourdoughs. Furthermore, doughs A and C also contained *Lb. rossiae* and *Lb. paralimentarius* respectively, but these species were detected only by use of DNA extracted with the protocol reported by Ampe, et al. (1999), probably since the detection level of this method is more sensitive than that of the method reported by Settanni, et al. (2005). These results were in agreement with those obtained with the cultivation method performed as previously described by Corsetti, et al. (2004), and they showed isolation of *Lb. plantarum* and *Lb. sanfranciscensis* from the 10^{-8} plate count. In contrast, *Lb. rossiae* and *Lb. paralimentarius* were not found; they were isolated from 10^{-7} and 10^{-6} plate counts, respectively.

In another approach called length heterogeneity PCR (LH-PCR), Müller, et al. (2001) used the variability in sequence length of rDNA amplicons to monitor the development of the flora in a type II sourdough fermentation performed at 40°C over five days to demonstrate the behavior of *Lb. pontis* and *Lb. amylovorus*. (Müller, et al. 2001; Vogel, et al. 1999). Despite a dominance of *Lb. pontis* at the beginning of the fermentation, after five days *Lb. amylovorus* clearly became the dominant species.

6 Culture Independent Methods to Analyze Sourdough Yeasts

A fungi-specific PCR-DGGE using 28S rDNA fragments was established to characterize the composition of three commercial starters, baker's yeast and an artificial mixture thereof, as well as rye flour in comparison to the development of the flora during sourdough fermentation (Meroth, et al. 2003). The analysis of the starter mixture revealed the presence of *C. humilis*, *Debaryomyces hansenii*, *S. cerevisiae*, and *S. uvarum*. For baker's yeast, DGGE bands of *D. hansenii*, *S. uvarum*, and *S. cerevisiae* were found. DGGE profiles of DNA of rye flour revealed three bands. One band could be identified as *S. cerevisiae*, but with the other bands, sequencing failed. Interestingly, these bands also appeared in the commercial starters and the starter mixture. With these starters four sourdoughs (A to D), propagated by repetitive back-slopping, were fermented. During the fermentation process, the yeast biota was monitored and changes in the DGGE profiles of all fermentation batches were observed within a few days, and unique profiles were obtained at the end of the propagation process after five days. *C. humilis* and *S. cerevisiae* predominated in doughs A (traditional process with rye flour at 25°C) and B (traditional process with rye flour at 30°C), respectively, and remained dominant until the end of fermentation. *Candida glabrata* was detected in batches C (traditional process with rye flour at 40°C) and D (process with rye bran at 40°C) at days 5 and 6, respectively, but decreased rapidly in numbers, as indicated by the disappearance of the band. Furthermore, *S. cerevisiae* was detected, with a faint band, at the end of fermentation in batches A, C, and D. Besides *S. cerevisiae*, *Issatchenkia orientalis* was also detected in batch D after two days of fermentation and dominated during the whole fermentation period.

Gatto and Torriani (2004) monitored the LAB and yeast flora of a traditional wheat sourdough (28°C, 24 hours) manufactured without the addition of baker's yeast, and using a long-period propagated homemade sourdough starter. Surprisingly, the newly developed 26S rDNA-based PCR-DGGE revealed no diversity in the yeast population. Only one band of increasing intensity throughout fermentation was visible and identified as *S. cerevisiae*. In contrast, the composition of the LAB flora in this sourdough was complex and changed during the fermentation period.

Culture-independent approaches can be superior to cultural methods with regard to identifying organisms hardly or not growing on commonly used growth media, and the time needed to analyze a sample. Nevertheless, they are afflicted

by some drawbacks that may limit an unrestricted interpretation of the data regarding quantification and flora composition. Sourdough is a highly complex matrix resulting in a reduced or undefined detection limit and a bias due to a less-than-optimal DNA isolation procedure. Co-migrating bands in DGGE analyses, e.g. reported for *Lb. casei* and *Lb. rhamnosus* (Gatto and Torriani 2004) or *Lb. pontis* and *Lb. sanfranciscensis* (Merøth, et al. 2003). Sometimes two bands for one organism occur, as is the case for *P. acidilactici*. Additional problems may arise from faint PCR-DGGE bands not resulting in sequences upon gel excision, purification and sequencing or failing comparison of migration distances of fragments with those of references. It is not possible to draw conclusions about the viability of detected organisms, as the target DNA may originate from living cells in a non-culturable state, dead cells or may be released from cells that are lysed during fermentation.

7 Identification of Sourdough LAB Using Molecular Methods

Although the extensive characterization of physiology and biochemistry is of great value in practice and application of these organisms, they became less important for classification and identification purposes. In view of the increasing number of more and more species – often closely related and physiologically elusive – the consequent use of molecular approaches is the reliable solution for classification and identification (Schleifer, et al. 1995). Choosing the right strategy depends on the problems to solve. To determine what microbial events are occurring in sourdoughs, it is necessary to understand the doubtless identification of pure cultures and the characterization of mixed microbial communities.

Gene probes and PCR assays. Direct detection and identification of isolates is performed by the use of specific gene-probes applicable in different formats (Blot-Hybridization, reverse Hybridization, PCR-assay). Many gene probes mainly targeting ribosomal RNA with specificity for different sourdough lactobacilli have been described (Hensiek, et al. 1992; Ehrmann, et al. 1994; Müller, et al. 2000a). Gene probes are especially useful for the identification of LAB which cannot be reliably differentiated by simple phenotypic tests or by standard procedures, since some are difficult to grown on conventional media (Vogel, et al. 1994). Specific oligonucleotides were also used in PCR assays for differentiation of *Lb. pontis*, *Lb. panis* and *Lb. frumenti* in a multiplex format (Müller, et al. 2000b). In PCR-based techniques the specificity of gene probe approaches are combined with an enormous sensitivity. DNA extracted from sourdough allowed the detection of 10^2 cfu/ml of *Lb. sanfranciscensis* (Zapparoli and Torriani 1997).

DNA-based typing methods. Different genotyping techniques have been applied to LAB in general and to sourdough LAB in particular. The major advantages of these DNA-based typing methods lie in their discriminatory power and in their universal applicability as no sequence information is necessary.

Molecular typing methods applicable to sourdough LAB include plasmid profiling, the analyses of fragment length polymorphisms (REA, RFLP, ribotyping), pulse-field gel electrophoresis (PFGE) and RAPD.

Plasmid profiling. Plasmid profiling was formerly considered suitable for the typing of individual strains within a species. Although in some LAB plasmids seem to occur very frequently in high numbers (e.g., *Lb. plantarum*), other typical sourdough species appear to be rather poor in plasmid content. Due to the lack of plasmids, and as a result of the instability of extrachromosomal DNA, methods that use chromosomal DNA, are superior to this technique in the respective species and have become more popular (Duffner and O'Connell 1995).

Restriction enzyme analysis/restriction fragment length polymorphism. REA involves the digestion of chromosomal DNA with restriction endonucleases. The selection of an appropriate restriction enzyme or a set of enzymes is important for obtaining revealing patterns. REA was applied successfully to differentiate between strains of *Lb. casei*, *Lb. rhamnosus* and *Lb. reuteri* (Stahl and Molin 1994; Áhrne and Molin 1997). The complexity of the banding pattern makes visual evaluation difficult, whereas blotting and hybridization with a gene probe targeted against rDNA (ribotyping) results in simpler patterns with remaining vital significance by visualizing restriction fragment length polymorphism (RFLP). Ribotyping has been used with some success to study the diversity of strains of *Lb. reuteri* and *Lb. fermentum* isolated from the mouse ileum (Ning, et al. 1997), and to characterize strains of different *Lactobacillus* species (Zohng, et al. 1998). The use of the insertion sequence IS153 previously isolated from *Lb. sanfranciscensis* allowed reliable discrimination of all tested isolates at strain level (Ehrmann and Vogel 2001).

Pulse-field gel electrophoresis. A highly discriminatory and reproducible method became known as PFGE and has been used to differentiate strains. This type of electrophoresis enables the separation of high-molecular-weight fragments generated by restriction of the genome to a low number of fragments. Zapparoli, et al. (1998) stated a high discriminatory power of PFGE indicating the existence of a genetical polymorphism in 11 strains of *Lb. sanfranciscensis*. The differentiation of 30 *L. plantarum* strains was achieved by REA-PFGE, that allowed the *Lb. plantarum* strains to be divided into 10 different genomic groups (Pepe et al., 2004).

Random amplified polymorphic DNA (RAPD). RAPD is a means of rapidly detecting polymorphisms for genetic fingerprinting (Welsh and McClelland 1990). This method has considerable appeal as it is faster and less expensive than other identification methods. Multiple studies using RAPD have been used to characterize species occurring in or isolated from sourdough (Johansson, et al. 1995; Hamad, et al. 1997; Hayford, et al. 1999; Ehrmann, et al. 2002; DeVuyst, et al. 2002; Du Toit, et al. 2003). This technique was revealed to be as discriminative as other species-specific methods. The typing efficiency is markedly increased when several single primers are applied. Zapparoli, et al. (1998) demonstrated a high degree of discriminatory power showing genetic polymorphism within *Lb. sanfranciscensis* depending on the primer used. The combination of RAPD profiles generated with different primers was also shown to enable

differentiation of sourdough *Lactobacillus* and *Weissella* strains (Corsetti, et al. 2003). RAPD-PCR was applied for the identification and typing of the LAB isolated from 25 samples of sourdoughs used for the production of traditional breads (*Carasau*, *Moddizzosu*, *Spianata*, *Zichi*) in Sardinia (Catzeddu, et al. 2006). In this study RAPD-PCR with a single primer followed by cluster analysis did not allow the identification of the isolates at the species level. However, a multidimensional scaling/bootstrapping approach on the RAPD-PCR patterns allowed the identification of 12 different species of LAB. *Lb. pentosus* dominated the lactic microflora of many samples while *Lb. sanfranciscensis* was isolated only from a limited number of samples. Although heterofermentative species represented between 30 percent and 60 percent of the isolates in *Carasau*, *Spianata* and *Zichi* sourdoughs, only 2 percent of the isolates from *Moddizzosu* sourdoughs were identified as heterofermentative LAB.

Amplified fragment length polymorphism (AFLP). As RAPD sometimes suffers from low reproducibility caused by variable conditions during PCR or different fidelity of polymerase used, the transfer of data from one laboratory to another and a set up of a universally usable database of patterns is difficult. A high reproducibility is given using the approach of Amplified Fragment Length Polymorphisms (AFLP). It was first introduced by Vos, et al. (1995) for plants and further used to analyze the diversity of clinically relevant bacteria (Valsangiacomo, et al. 1995). AFLPs were shown by several authors to be applicable to LAB differentiating at and below species level Gancheva, et al. (1999) performed a polyphasic approach towards the identification of strains belonging to the *Lb. delbrueckii* - group. AFLP typing was compared with results of SDS-PAGE of whole cell proteins and RAPD typing. The discriminatory power of AFLP-derived patterns was comparable with the others and even species like *Lb. gasseri* and *Lb. johnsonii*, which could not be separated by SDS-PAGE before, could be differentiated by AFLP. The clear allocation of the recently described new species *Lb. amylolyticus* and *Lb. iners* into new clusters, demonstrated the applicability of AFLP to identify new taxons. Kunene and co-workers (Kunene, et al. 2000) applied AFLP to distinguish strains of *Lb. plantarum* and *Lc. mesenteroides* from different sorghum doughs. Torriani, et al. (2001) used both RAPD and AFLP to discriminate the closely related species *Lb. plantarum*, *Lb. pentosus* and *Lb. paraplantarum* at the infraspecies level. There was a good agreement between the two genetic approaches, but between genomically highly related strains varying levels of similarity were obtained.

8 Identification of Sourdough Yeasts Using Molecular Methods

Most reports about sourdough microflora have focused on the characterization of lactobacilli and their role during fermentation, while a smaller number of papers have been devoted to investigate the yeast component, presumably because baker's yeast is widely used in sourdough bread technology.

The traditional systematics and identification of yeasts have been based on biochemical tests, and morphological and physiological criteria. Taxonomic problems arise as imperfect fungi cannot be studied using traditional genetics, and physiological features significantly alter when changes occur in growth conditions. Thus, molecular characteristics define and change current yeast taxonomy.

Typing methods (karyotyping, PFGE, PCR-RFLP, RAPD) as well as comparative sequence analysis proved high potential when classifying sourdough yeasts. Markers of interest are rRNA genes, internal transcribed spacer (ITS), elongation factor 3 (EF-3) or others.

A polyphasic approach including PFGE (karyotyping), PCR-RFLP of 18S rRNA genes and EF-3 and metabolic activities (Biolog system) was used to characterize the yeasts in five sourdoughs of major bakeries in Finland (Mäntynen, et al. 1999). The authors found that most of the commercial sourdoughs formed their own unique RFLP-cluster that contained yeasts which were similar to *C. milleri* and *S. exiguus*. Whereas the Biolog system in this study identifies poorly or misidentified yeast strains, DNA-based methods provided more information to identify bakery strains at species level.

Sourdoughs sampled from Sicilian bakeries that did not use starter cultures were investigated with PFGE, PCR-RFLP of non-transcribed spacer 2, the inter - δ region, ITS regions of the ribosomal DNA and partial sequencing of the 26S RNA gene sequences (Pulvirenti, et al. 2001). Based on these techniques the authors infer that the yeast species most frequent in Sicilian sourdoughs are in the following order: *S. cerevisiae*, *I. orientalis*, *C. milleri* and *C. humilis*. Additionally, from this study it became clear that the choice of the method to be used must be carefully evaluated on the basis of the genus and species to be identified. To differentiate *C. humilis* from *C. milleri* (100 percent similarity of diagnostic D1/D2 region of the 26S rRNA gene) was only possible by PCR-RFLP of the ITS region.

Pulvirenti, et al. (2004) analyzed the occurrence and dominance of yeasts in homemade sourdoughs (wheat flour) taken from small bakeries in Sicily. Identification of 180 strains through PCR-RFLP based on ITS regions showed that the majority of sourdoughs (80 percent) were dominated by one single species, of which 91 percent were *S. cerevisiae*, while *C. humilis*, *I. orientalis* and *S. exiguus* were only dominant in 3 percent. Interestingly, a restriction analysis of the inter- δ region allowed the identification of a high polymorphism in *S. cerevisiae*, even in strains from same samples, which means that up to five different strains of a species co-exist in dominant cell numbers in one sourdough.

9 Ecological Prevalence

LAB occurring in sourdough can be allotted to three groups, which depict features of “autochthonous,” “ubiquitous” or “allochthonous” bacteria with respect to the sourdough environment. Among these only *Lb. sanfranciscensis* has exclusively been isolated from sourdoughs up to now. For the moment this holds true also for

the very recently described species *Lb. frumenti*, *Lb. mindensis* and *Lb. spicheri*. (Müller, et al. 2000a; Ehrmann, et al. 2003; Meroth, et al. 2004). Whenever these organisms can be considered typical for sourdoughs, it remains unclear whether an “autochthonous” sourdough flora exists or one should search for it. It seems possible that “inoculation” of cereals used for dough originates from the feces of mice in elevators. As legend has it cow and horse dung was employed for traditional Italian panettone. Thus, *Lb. reuteri* and *Lb. pontis* described as competitive parts of the sourdough microflora could be considered part of the “autochthonous” for these environments while they colonize other habitats in mouse, pig and possibly other intestinal sources (Du Troit, et al. 2003).

Within *Lb. plantarum* and *Lb. brevis* a big versatility in potential or metabolic traits is found. Therefore, it is not surprising that specific strains of these “ubiquitous” species were isolated as a competitive part of the flora.

Finally, and although often found, *Lactococcus lactis* may be considered a deliberately used, and therefore “allochthonous,” organism in sourdoughs which can transiently be competitive, though is not competitive in back-slopping procedures. *L. lactis* and *Lb. plantarum* are probably the best studied LAB also in their genetics, and genome sequences are available for them. A draft genome sequence is currently established for *Lb. brevis*. However, none of the strains chosen for these approaches originates from sourdough as the general importance of these genera lies in other fields.

10 Role of Molecular Methods to Explain Sourdough Ecology

Apart from following sourdough bacteria along taxonomic markers the forthcoming value of molecular methods in understanding the sourdough ecosystem resides in monitoring of their metabolic traits on the level of their genomic presence and regulation and elucidating their ecological roles by using knock out mutants and over-expressing strains. The ecological parameters cross-talking with the genetic setting of the microorganisms dictate their presence and roles in the sourdough. While most research was targeted on monitoring bacteria/yeasts in a (food) ecosystem along their taxonomic/phylogenetic status, looking at these organisms as “metabolic groups” may provide a different type of insight in understanding these ecosystems. The success of these approaches is strongly dependent on thorough knowledge of their genome structure and flexibility, genetic amenability and gene regulation, which is, therefore, briefly discussed here.

11 Genome Structure and Flexibility

Molecular analysis techniques provide possibilities to determine genome sizes, structures and flexibility based on the presence of mobile genetic elements of the sourdough bacteria. Investigations using pulsed field electrophoreses on the

genome sizes of sourdough LAB are scarce, indicating relatively small genomes for *Lb. sanfranciscensis* (Zapparoli, et al. 1998), and *Lb. pontis* and *Lb. reuteri* in the range of 1.5 Mbp to 2 Mbp. This indicates that the metabolic potential and adaptation to several ecological niches of these species may be limited, whereas they appear as specialized to be competitive in specific cereal-related environments. The genomes of *Lb. pontis*, *Lb. reuteri* and *Lb. fermentum* are high in G+C (48 percent to 50 percent) while the genome of *Lb. sanfranciscensis* exhibits a low G+C content with 36 percent to 38 percent (Siranganathan, et al. 1985). Whereas the occurrence of multiple plasmids is common in *Lb. reuteri* and *Lb. plantarum*, among 100 strains of *Lb. sanfranciscensis* strains only a few were found to contain plasmids. In *Lb. sanfranciscensis* strains generally one or even no plasmid can be found (Reinkemeier and Röcken 1995; authors' observation). Lönner, et al. (1990) reported that strains of *Lb. acidophilus*, *Lb. plantarum*, *Lb. sanfranciscensis* and *Lb. alimentarius* freshly isolated from sourdoughs often harbored a high number of plasmids with molecular weights from 1.5 to 43 MDal. In contrast type strains of these species, which typically have undergone repeated propagation in laboratory media, often contained few or no plasmids. If plasmids are lost upon transfers in laboratory media, they may harbor genes for metabolic traits only relevant in the adaptation to the environment they were isolated from. A similar situation is found in *Lb. pontis*. Furthermore, insertion elements were characterized in *Lb. sanfranciscensis* that appear to be frequently present in all strains (Ehrmann and Vogel 2001). While they are useful in strain typing, their natural role in genetic flexibility or their use for mutant construction remains to be elucidated. Some phages capable to lyse *Lb. sanfranciscensis* strains have been described (Foschino, et al. 2003; 2005). In another study several lytic phages were characterized for *Lb. fermentum* (Foschino, et.al. 2001).

12 Genetic Amenability of Sourdough LAB

The almost general absence of plasmids may partly explain the low transformation efficiencies observed with strains of the genera isolated from sourdough, and considered as “autochthonous” in the above sense, as a lack in plasmid replication mechanisms. However, the transformable type strain *Lb. sanfranciscensis* DSM 20451 harbors no detectable plasmid. Transformation via electroporation was achieved with few strains only at rates of $< 10^6$ transformants per μg plasmid DNA used. Typical recognized replicons originated from *Lb. plantarum*, e. g. in pLP3537. For *Lb. sanfranciscensis*, *Lb. pontis* and *Lb. reuteri* there are principal demonstrations that chromosomal integration of plasmid DNA and construction of knock out mutants by the use of plasmid vectors non-replicative in these LAB is possible. A levansucrase negative mutant of *Lb. sanfranciscensis* was constructed by insertional inactivation of the corresponding gene that lost the ability to hydrolyze sucrose, and did not produce fructan or 1-kestose (Tieking, et al. 2005). For *Lb. reuteri* in vivo expression technology (IVET) has been established to isolate

genes specifically expressed in the intestinal tract (Walter, et al. 2003). The availability of genome sequences from related LAB, namely *Lb. plantarum* and *L. lactis*, have opened up broad possibilities for PCR strategies based on conserved regions of genes of interest. Holistic approaches using comparative proteomics and reverse genetics have been used to describe stress reactions of *Lb. pontis* (Steudel 2001) and *Lb. sanfranciscensis* (Scheyhing 2003).

13 Gene Cloning and Characterization

The characterization of genes in sourdough LAB contributes to the elucidation of the function of specific metabolic traits for the organisms in the sourdough environment. A variety of genes has been cloned and characterized from LAB species found in sourdoughs. However, most of the strains used in these approaches were originally isolated from environments other than sourdough comprising “ubiquitous” or “allochthonous” genera. These include a vast number of genes from *L. lactis* and *Lb. plantarum* which shall not be referred to here, as well as genes encoding specific traits of whose function can only partly be related to the sourdough environment. Examples for respective genes are galactose and xylose uptake and isomerization (Bor, et al. 1992; Chaillou, et al. 1998; Djordjevicet, et al. 2001), or genes for the S-layers (Kahala, et al. 1997; Jakava-Viljanen, et al. 2002), tyrosin decarboxylase (Lucas and Lonvaud-Funel 2002) and glutamate racemase (Yagasaki, et al. 1995) of *Lb. brevis*, cystathionine- γ -lyase of a guinea pig vaginal isolate of *Lb. fermentum* (Turner, et al. 1997; Turner, et al. 1999). These enzymes play roles in competitiveness and survival, formation of biogenic amines, or aroma precursor formation, respectively.

Within the autochthonous sourdough lactobacilli, namely *Lb. sanfranciscensis* and *Lb. reuteri*, a major focus has been on the characterization of genes encoding glycosyltransferases. The exopolysaccharides formed by these bacteria may serve ecological aspects but, apart from this, have major impacts on food textures when added or produced *in situ* by starter strains (Tieking, et al. 2003; Tieking and Gänzle 2005; Lacaze, et al. 2007). A comprehensive overview on these enzymes was given by Korakli and Vogel (2006). Recent work was furthermore directed to characterize genes involved in the formation of aroma precursors.

Lb. pontis can metabolize arginine, forming the aroma precursor ornithine which is converted to 2-acetyl-1-pyrroline, which is mainly responsible for the roast aroma of bread. The arginine pathway was cloned, the arginine deiminase was knocked out and it was demonstrated that ornithine formation, indeed, is the key feature for roast aroma in bread (personal communication by Hertel, Universität Hohenheim, Germany). The genes of the cystathionine- γ -lyases of several *Lb. pontis* strains and other sourdough lactobacilli were cloned aiming at elucidation of the impact of the methionine pathway in bread flavor development and dough (Vermeulen, et al. 2003).

The clear emphasis on genetic studies within the typical sourdough lactobacilli is on *Lb. sanfranciscensis*. Most genes involved in the central carbon metabolism have been cloned and characterized, the recombinant proteins have been expressed and purified, and their kinetic properties determined (Ehrmann and Vogel 1998; Knorr, et al. 2001a, b). Among these, two enzymes and their genes deserve special mention. Phosphoketolase and 6-phospho-gluconate dehydrogenase are key enzymes in the heterofermentative pathway. The phosphoketolase of *Lb. sanfranciscensis* serves two reactions: splitting xylulose-5-phosphate in the heterofermentative pathway, and fructose-6-phosphate in direction of erythritol production. Furthermore, genes involved in the utilization of peptides resulting from the activity of the flour proteases on cereal proteins were characterized, and the proteolytic system of *Lb. sanfranciscensis* was described (Vermeulen, et al. 2005). The knowledge of genes involved in carbon and peptide utilization enables screening of strains for competitiveness and aroma (precursor) formation from the preferentially uptaken peptides upon their intracellular cleavage to amino acids and subsequent catabolism.

14 Gene Regulation

The emerging genomic data on bacterial species found in sourdough enables global regulation studies on the reaction of these bacteria towards environmental stresses or the adaptation to substrates and other environmental changes. Again, most of the studies performed with such tools were performed with *L. lactis* and *Lb. plantarum*, and shall not be discussed here to keep the focus of this chapter. Despite the absence of a complete genome sequence enabling genome-wide transcriptome analyses with the autochthonous group of sourdough LAB, gene regulation was studied on the level of the proteome and transcriptome of *Lb. sanfranciscensis*. Extensive studies have been performed on the stress reaction of *Lb. sanfranciscensis* by differential proteomics comparing heat, salt, high pressure and cold stress with optimally growing cells (Scheyhing 2003; De Angelis 2003; Pavlovic, et al. 2005; Hörmann, et al. 2006). In these approaches several general and specific stress proteins have been identified.

15 Conclusions and Trends

A considerable arsenal of powerful molecular techniques exists that allows detection, characterization and monitoring of LAB in sourdoughs. The analysis of many artisanal sourdoughs during recent years has led to the description of several new species whose role in the respective fermentations mostly remained unclear. Nevertheless, these isolates constitute a large genetic pool of interesting organisms and traits thereof, which are waiting for exploitation. The upcoming possibilities of

DGGE and (micro)array analyses enable the easy monitoring of microbiota in sourdough fermentations and understanding of their metabolism and adaptation to a genomic level. As many of these bacteria share sourdough and intestinal environments they are very interesting candidates for applications as probiotics or therapeutics of intestinal diseases. To date, sufficient data for deliberate and targeted applications are available for comparably few strains of the vast sourdough microbiota. By the use of molecular techniques, namely holistic ones as all kinds of omics, a knowledge base can rapidly be established to demonstrate selective criteria for sourdough strains to obtain a status of qualified presumption of safety (QPS) as proposed by the European Food Safety Authority (EFSA) (Anonymous 2005). In this view, isolation from a traditional environment or human should not be considered to deliver such safe strains, but safety must be proven along the absence or presence of specific genetic and metabolic properties. Establishment of a respective knowledge base, therefore, is becoming a key issue to make use of these organisms as life food constituents beyond their sourdough application in non-baked goods. De Angelis, et al. (2007) have employed a polyphasic approach to investigate strains of *Lb. sanfranciscensis* along their metabolic potential. This study is an example demonstrating an approach to generate a knowledge base on sourdough LAB and understand their role in this fermentation, enabling the purposive design of improved processes and products. The reference of metabolic traits investigated here to their genetic background shall facilitate screening of a large variety of isolates with fast genetic tools and select them along their genetic configuration, which determines their metabolic potential.

References

- Aarnikunnas, J., von Weymarn, N., Ronnholm, K., Leisola, M., Palva, A. 2003. Metabolic engineering of *Lactobacillus fermentum* for production of mannitol and pure L-lactic acid or pyruvate. *Biotechnol. Bioeng.* 82, 653–663.
- Ahrné, S., Molin, G. 1997. Restriction endonuclease analysis of total chromosomal DNA of *Lactobacillus*. *Microecol. Therapy*, 26, 27–30.
- Ampe, F., Miambi, E. 2000. Cluster analysis, richness and biodiversity indexes derived from denaturing gradient gel electrophoresis fingerprints of bacterial communities demonstrate that traditional maize fermentations are driven by the transformation process. *Int. J. Food. Microbiol.* 60, 91–97.
- Ampe, F., N. ben Omar, C. Moizan, C. Wachter, J.-P. Guyot. 1999. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *App. Environ. Microbiol.* 65, 5464–5473.
- Ampe, F., Sirvent, A., Zakhia, N. 2001. Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. *Int. J. Food Microbiol.* 65, 45–54.
- Anonymous, 2005. Opinion of the Scientific Committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives, *The EFSA Journal* 226, 1–12.

- Antuna, B., Martinez-Anaya, M.A. 1993. Sugar uptake and involved enzymatic activities by yeasts and lactic acid bacteria: their relationship with bread making quality. *Int. J. Food Microbiol.* 18, 191–200.
- Aslam, Z., Im, W.T., Ten, L.N., Lee, M.J., Kim, K.H., Lee, S.T. 2006. *Lactobacillus siliginis* sp. nov., isolated from wheat sourdough in South Korea. *Int. J. Syst. Evol. Microbiol.* 56, 2209–13.
- Axelsson, L. 1998. Lactic Acid Bacteria: Classification and Physiology In: Salminen, S., van Wright, A. (Eds.), *Lactic Acid Bacteria*. New York: Marcel Dekker.
- Axelsson, L.T., Ahrne, S. 2000. Lactic Acid Bacteria. In: Priest, F.G., Goodfellow, M., *Applied Microbial Systematics*. Kluwer Academic Publishers.
- Azar, N. Ter-Sarkissian, H. Ghavifek, T. Ferguson, Ghassemi, H. Microbiological aspects of Sangak bread. 1977. *J. Food Sci. Technol.* 14, 251–254.
- Barber, S., Báguena, R. 1988. Microflora de la masa panaria. V. Aislamiento, identificación y evaluación de las propiedades funcionales de microorganismos de la masa madre. *Rev. Agr. Tecnol. Alim.* 28, 67–78.
- Barber, S., Báguena, R., Martinez-Anaya, M.A., Torner, M.J. 1983. Microflora de la masa madre panaria. I. Identificación y propiedades funcionales de microorganismos de masas madre industriales, elaboradas con harina de trigo. *Rev. Agr. Tecnol. Alim.* 23, 552–562.
- Bonnarme, P., Lapadatescu, C., Yvon, M., Spinnler, H. E. 2001. L-methionine degradation potentialities of cheese-ripening microorganisms. *J. Dairy Sci.* 68, 663–74.
- Bor, Y.C., Moraes, C., Lee, S.P., Crosby, W.L., Sinskey, A.J., Batt, C.A. 1992. Cloning and sequencing the *Lactobacillus brevis* gene encoding xylose isomerase. *Gene*, 114, 127–132.
- Boraam, F., Faid, M., Larpant, J.P., Breton, A. 1993. Lactic acid bacteria and yeast associated with traditional Moroccan sourdough bread fermentation. *Sci. Aliment.* 13, 501–509.
- Cai, Y., Okada, H., Mori, H., Benno, Y., Nakase, T. 1999. *Lactobacillus paralimentarius* sp. nov., isolated from sourdough. *Int. J. Syst. Bacteriol.*, 49, 1451–1455.
- Catzeddu, P., Mura, E., Parente, E., Sanna, M., Farris, G.A. 2006. Molecular characterization of lactic acid bacteria from sourdough breads produced in Sardinia (Italy) and multivariate statistical analyses of results. *Syst. Appl. Microbiol.* 29, 138–44.
- Chaillou, S., Bor, Y.C., Batt, C.A., Postma, P.W., Pouwels, P.H. 1998. Molecular cloning and functional expression in *Lactobacillus plantarum* 80 of *xylT*, encoding the D-xylose-H⁺ symporter of *Lactobacillus brevis*. *App. Environ. Microbiol.* 64, 4720–4728.
- Collar, C., Andreu, P., Martínez-Anaya, M.A. 1998. Interactive effects of flour, starter and enzyme on bread dough machinability. *Z. Lebensm. Unters. Forsch. A* 207, 133–139.
- Collins, M.D., Rodrigues, U., Ash, C. 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. *FEMS Microbiol. Lett.* 77, 5–12.
- Corsetti, A., Settanni, L., van Sinderen, D., Felis, G.E., Dellaglio, F., Gobbetti, M. 2005. *Lactobacillus rossii* sp. nov., isolated from wheat sourdough. *Int. J. Syst. Evol. Microbiol.* 55, 35–40.
- Corsetti, A., De Angelis, M., Dellaglio, F., Paparella, A., Fox, P. F., Settanni, L., Gobbetti, M. 2003. Characterization of sourdough lactic acid bacteria based on genotypic and cell-wall protein analyses. *J. Appl. Microbiol.* 94, 641–651.
- Corsetti, A., Settanni, L., Van Sinderen, D. 2004. Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their *in vitro* and *in situ* activity. *J. Appl. Microbiol.* 96, 521–534.
- Corsetti, A., Gobbetti, M., Balestrieri, F., Paoletti, F., Russi, L., Rossi, J. 1998. Sourdough lactic acid bacteria effects on bread firmness and staling. *J. Food Sci.* 63, 347–351.
- Corsetti, A., Lavermicocca, P., Morea, M., Baruzzi, F., Tosti, N., Gobbetti, M., 2001. Phenotypic and molecular identification and clustering of lactic acid bacteria and yeasts from wheat (species *Triticum durum* and *Triticum aestivum*) sourdoughs of southern Italy. *Int. J. Food. Microbiol.* 64, 95–104.

- Damiani, P., Gobbetti, M., Cossignani, L., Corsetti, A., Simonetti, M.S., Rossi, J. 1996. The sourdough microflora: characterization of hetero- and homofermentative lactic acid bacteria, yeasts, and their interactions on the basis of the volatile compounds produced. *Lebensm. Wiss. Technol.* 29, 63–70.
- De Angelis, M. 2003. Environmental stress responses in sourdough lactic acid bacteria. In *Second International Symposium on Sourdough, From Fundamentals to Applications*, Brussels, 8–11 October 2003.
- De Angelis, M., Di Cagno, R., Gallo, G., Curci, M., Siragusa, S., Crecchio, C., Parente, E., Gobbetti, M. 2007. Molecular and functional characterization of *Lactobacillus sanfranciscensis* strains isolated from sourdoughs. *Int. J. Food Microbiol.* 114, 69–82.
- De Vuyst, L., Schrijvers, V., Paramithiotis, S., Hoste, B., Vancanneyt, M., Swings, J., Kalantzopoulos, G., Tsakalidou, E., Messens, W. 2002. The biodiversity of lactic acid bacteria in Greek traditional wheat sourdoughs is reflected in both composition and metabolite formation. *App. Environ. Microbiol.* 68, 6059–6069.
- Djordjevic, G.M., Tchieu, J.H., Saier, M.H. 2001. Genes involved in control of galactose uptake in *Lactobacillus brevis* and reconstitution of the regulatory system in *Bacillus subtilis*. *J. Bacteriol.* 183, 3224–3236.
- Du Toit, M., Dicks, L.M.T., Holzapfel, W.H. 2003. Identification of heterofermentative lactobacilli isolated from pig feces by numerical analysis of total soluble cell protein and RAPD-patterns. *Lett. Appl. Microbiol.* 37, 12–16.
- Duffner, F., O'Connell, M. 1995. Comparative evaluation of plasmid profiling and ribotyping in the analysis of *Lactobacillus plantarum* strain heterogeneity in silage. *J. Appl. Bacteriol.* 78, 20–7.
- Ehrmann M.A., Vogel, R.F. 1998. Maltose metabolism of *Lactobacillus sanfranciscensis*: cloning and heterologous expression of the key enzymes, maltose phosphorylase and phosphoglucomutase. *FEMS Microbiol. Lett.* 169, 81–86.
- Ehrmann, M.A., Brandt, M., Stolz, P., Vogel, R.F., Korakli, M. 2007. *Lactobacillus secaliphilus* sp. nov., isolated from type II sourdough fermentation, *Int. J. Syst. Evol. Microbiol.* 57, 745–50.
- Ehrmann, M.A., Ludwig, W., Schleifer K.H. 1994. Reverse Dot blot hybridization: a useful method for the direct identification of lactic acid bacteria in fermented food. *FEMS Microbiol. Lett.* 117, 143–150.
- Ehrmann, M.A., Müller, M.R.A., Vogel, R.F. 2003. *Lactobacillus mindensis*, spec. nov., isolated from sourdough fermentation. *Int. J. Syst. Evol. Microbiol.* 53, 7–13.
- Ehrmann, M.A., Vogel, R.F. 2001. Characterization of IS153, an IS3-family insertion sequence isolated from *Lactobacillus sanfranciscensis* and its use for strain differentiation. *Syst. Appl. Microbiol.* 24, 443–450.
- Ehrmann, M.A., Vogel, R.F. 2005. Molecular taxonomy and genetics of sourdough lactic acid bacteria. *Trends Food Sci. Technol.* 16, 31–42.
- Felis, G.E., Dellaglio, F., Mizzi, L., Torriani, S. 2001. Comparative sequence analysis of a recA gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. *Int. J. Syst. Evol. Microbiol.* 51, 2113–7.
- Foschino, D., Galli, A. 1997. Italian style of life: pane, amore e ... lievito naturale! *Tecnologie alimentari* 1, 42–59.
- Foschino, R., Galli, A., Perrone, F., Ottogalli, G. 1995. Prodotti da forno ottenuti da impasti a lievitazione naturale-studio e caratterizzazione della microflora tipica. *Tecnica Molitoria*, 5, 257–279.
- Foschino, R., Picozzi, C., Galli, A. 2001. Comparative study of nine *Lactobacillus fermentum* bacteriophages. *J. Appl. Microbiol.* 91, 394–403.
- Foschino, R., Picozzi, C., Gallina, S., Scarpellini, M., Venturelli, E., Galli, A. 2003. Bacteriophages of sourdough lactobacilli: Isolation, characterization and lysogeny. In *Second International Symposium on Sourdough, From Fundamentals to Applications*, Brussels, 8–11 October 2003.
- Foschino, R., Venturelli, E., Picozzi, C. 2005. Isolation and characterization of a virulent *Lactobacillus sanfranciscensis* bacteriophage and its impact on microbial population in sourdough. *Curr. Microbiol.* 51, 413–8.

- Galli, A., Franzetti, L., Fortina, M.G. 1988. Isolation and identification of sour dough microflora. *Microbiologie-Aliments-Nutrition* 6, 345–351.
- Galli, A., Ottogalli, G. 1973. Aspetti della microflora degli impasti panettone. *Annali di Microbiologia e Enzimologia* 23, 39–49.
- Gancheva, A., Pot, B., Vanhonacker, K., Hoste, B., & Kersters, K. 1999. A polyphasic approach towards the identification of strains belonging to *Lactobacillus acidophilus* and related species. *Syst. Appl. Microbiol.* 22, 573–585.
- Gatto, V., Torriani, S. 2004. Microbial population changes during sourdough fermentation monitored by DGGE analysis of 16S and 26S rRNA gene fragments. *Ann. Microbiol.* 54, 31–42.
- Germond, J.E., Lapiere, L., Delley, M., Mollet, F., & Dellaglio, F. 2003. Evolution of the bacterial species *Lactobacillus delbrueckii*: A partial genomic study with reflections on prokaryotic species concept. *Mol. Biol. Evol.* 20, 93–104.
- Giraffa, G., Neviani, E. 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *Int. J. Food. Microbiol.*, 67, 19–34.
- Gobbetti, M. 1998. Interactions of lactic acid bacteria and yeasts in sourdoughs. *Trends Food Sci. Technol.* 9, 267–274.
- Gobbetti, M., Corsetti, A., Rossi, J. 1994b. The sourdough microflora. Interaction between lactic acid bacteria and yeasts: metabolism of carbohydrates. *World J. Microbiol. Biotechnol.* 10, 275–279.
- Gobbetti, M., Corsetti, A., Rossi, J., La Rosa, F. De Vincenzi, S., 1994. Identification and clustering of lactic acid bacteria and yeasts from wheat sourdoughs of central Italy, *Ital. J. Food Sci.* 6, 85–94.
- Grossilier, A., Lonvaud-Funel, A. 1999. Comparison of partial malolactic enzyme gene sequences for phylogenetic analysis of some lactic acid bacteria species and relationships with the malic enzyme. *Int. J. Syst. Bacteriol.* 4, 1417–28.
- Gullo, M., Romano, A.D., Pulvirenti, A., Giudici, P. 2002. *Candida humilis*—dominant species in sourdoughs for the production of durum wheat bran flour bread. *Int. J. Food Microbiol.* 80, 55–59.
- Hammad, S.H., Dieng, M.C., Ehrmann, M.A., Vogel, R.F. 1997. Characterization of the bacterial flora of Sudanese sorghum flour and sorghum sourdough. *J. Appl. Microbiol.* 83, 764–770.
- Hansen, B., Hansen, A. 1994. Volatile compounds in wheat sourdoughs produced by lactic acid bacteria and sourdough yeasts. *Z. Lebensm. Unters. Forsch.* 198, 202–209.
- Hayashi, N., Ito, M., Horiike, S., Taguchi, H. 2001. Molecular cloning of a putative divalent-cation transporter gene as a new genetic marker for the identification of *Lactobacillus brevis* strains capable of growing in beer. *Appl. Microbiol. Biotechnol.* 55, 596–603.
- Hayford, A.E., Petersen, A., Vogensen, F.K., Jakobsen, M. 1999. Use of conserved randomly amplified polymorphic DNA (RAPD) fragments and RAPD pattern for characterization of *Lactobacillus fermentum* in Ghanaian fermented maize dough. *App. Environ. Microbiol.*, 65, 3213–3221.
- Heilig, H.G.H.J., Zoetendahl, E.G., Vaughan, E.E., Marteau, P., Akkermans, A.D. L., De Vos, W.M. 2002. Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *App. Environ. Microbiol.*, 68, 114–123.
- Hensiek, R., Krupp, G., Stackebrandt, E. 1992. Development of diagnostic oligonucleotide probes for four *Lactobacillus* species occurring in the intestinal tract. *Syst. Appl. Microbiol.* 15, 123–128.
- Hörmann, S., Ehrmann, M.A., Vogel, R. F. 2006. Stress response of *Lactobacillus sanfranciscensis* DSM 20451^T: a comparative proteomic study between high pressure and other stresses. *Proteomics* 6, 1878–85.
- Infantes, M., Tourneur, C. 1991. Etude de la flore lactique de levains naturels de panification provenant de différentes régions françaises. *Sci. Alim.* 11, 527–545.
- Iorizzo, M., Coppola, R., Sorrentino, L., Grazia, L. 1995. Caratterizzazione microbiologica di paste acide molisane. *Ind. Aliment.* XXXIV, 1290–1294.
- Jakava-Viljanen, M., Avall-Jaaskelainen, S., Messner, P., Sleytr, U.B., Palva, A. 2002. Isolation of three new surface layer protein genes (*slp*) from *Lactobacillus brevis* ATCC 14869 and char-

- acterization of the change in their expression under aerated and anaerobic conditions. *J. Bacteriol.* 184, 6786–6795.
- Johansson, M.L., Quednau, M., Molin, G., Ahrné, S. 1995. Random amplified polymorphic DNA for rapid typing of *Lactobacillus plantarum* strains. *Lett. Appl. Microbiol.* 21, 155–159.
- Kahala, M., Savijoki, K., Palva, A. 1997. In vivo expression of the *Lactobacillus brevis* S-layer gene. *J. Bacteriol.* 179, 284–286.
- Keswani, J., Whitman, W.B. 2001. Relationship of 16S rRNA sequence similarity to DNA hybridization in prokaryotes. *Int. J. Syst. Evol. Microbiol.* 51, 667–678.
- Kline L., Sugihara, T.F. 1971. Microorganisms of the San Francisco sourdough bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. *Appl. Microbiol.* 21, 459–465.
- Knorr, R., Ehrmann, M.A., Vogel, R.F. 2001. Cloning of the phosphotransacetylase gene from *Lactobacillus sanfranciscensis* and characterization of its gene product. *J. Basic Microbiol.* 41, 339–349.
- Knorr, R., Ehrmann, M.A., Vogel, R.F. 2001. Cloning, expression, and characterization of acetate kinase from *Lactobacillus sanfranciscensis*. *Microbiol. Res.* 156, 1–11.
- Korakli, M., Vogel, R.F. 2006. Structure/function relationship of homopolysaccharide producing glycanucrases and therapeutic potential of their synthesized glycans. *Appl. Microbiol. Biotechnol.* 71, 790–803.
- Korakli, M., Gänzle, M.G., Vogel, R.F. 2002. Metabolism by *Bifidobacteria* and lactic acid bacteria of polysaccharides from wheat and rye and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J. Appl. Microbiol.* 92, 958–965.
- Kralj, S., van Geel-Schutten, G.H., Rahaoui, H., Leer, R.J., Faber, E.J., van der Maarel, M.J., Dijkhuizen, L. 2002. Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with alpha-(1→4) and alpha-(1→6) glucosidic bonds. *App. Environ. Microbiol.*, 68, 4283–4291.
- Kunene, N. F., Geornarsi, I., von Holy, A., Hastings J. W. 2000. Characterization of lactic acid bacteria from sorghum-based fermented weaning food by analysis of soluble proteins and AFLP fingerprinting. *App. Environ. Microbiol.*, 66, 1084–1092.
- Lacaze, G., Wick, M., Cappelle, S. 2007. Emerging fermentation technologies: Development of novel sourdoughs. *Food Microbiol.* 24, 155–160.
- Lin, C.F., Chung, T.C. 1999. Cloning of erythromycin-resistance determinants and replication origins from indigenous plasmids of *Lactobacillus reuteri* for potential use in construction of cloning vectors. *Plasmid*, 42, 31–41.
- Lin, C.F., Fung, Z.F., Wu, C.L., Chung, T.C. 1996. Molecular characterization of a plasmid-borne (pTC82) chloramphenicol resistance determinant (cat-TC) from *Lactobacillus reuteri* G4. *Plasmid*, 36, 116–124.
- Lönnner, C., Preve-Åkesson, K., Ahrné, S. 1990. Plasmid contents of lactic acid bacteria isolated from different types of sourdoughs. *Curr. Microbiol.* 20, 201–207.
- Lucas, P., Lonvaud-Funel, A. 2002. Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. *FEMS Microbiol. Lett.* 211, 85–89.
- Ludwig, W., Strunk, O., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M., Schleifer, K.H. 1998. Bacterial phylogeny based on comparative sequence data. *Electrophoresis*, 19, 554–568.
- Mäntynen, V.H., Korhola, M., Gudmundsson, H., Turakainen, H., Alfredsson, G.A., Salovaara, H., Lindström, K. 1999. A polyphasic study on the taxonomic position of industrial sour dough yeasts. *Syst. Appl. Microbiol.* 22, 87–96.
- Martinez-Anaya, M.A., Pitarch, B., Bayarri, P., de Barber, C.B. 1990a. Microflora of the sourdoughs of wheat flour bread. X. Interactions between yeasts and lactic acid bacteria in wheat doughs and their effects on bread quality. *Cereal Chem.* 67, 85–91.
- Martinez-Anaya, M.A., Torner, M.J., de Barber, B.C. 1990b. Microflora of the sourdough of wheat flour bread. XIV. Changes in volatile compounds during fermentation of doughs prepared with pure microorganisms and their mixtures. *Z. Lebensm. Unters. Forsch.* 190, 126–131.

- Meroth, C.B., Hammes, W.P., Hertel, C. 2004. Characterization of the microbiota of rice sourdoughs and description of *Lactobacillus spicheri* sp. nov. Syst. Appl. Microbiol. 27, 151–159.
- Meroth, C.B., Walter, J., Hertel, C., Brandt, M., Hammes, W.P. 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. App. Environ. Microbiol., 69, 475–482.
- Meroth, C.B., Hammes, W.P., Hertel, C. 2003. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. App. Environ. Microbiol. 69, 7453–61.
- Müller, M.R.A., Ehrmann, M. A., Vogel, R. F. 2000a. *Lactobacillus frumenti* sp. nov., a new lactic acid bacterium isolated from rye fermentations with a long fermentation period. International Journal of Syst. Evol. Microbiol. 50, 2127–2133.
- Müller, M.R.A., Ehrmann, M.A., Vogel R.F. 2000b. Multiplex PCR for the detection of *Lactobacillus pontis* and two related species in a sourdough fermentation. App. Environ. Microbiol., 66, 2113–2116.
- Müller, M.R.A., Wolfrum, G., Stolz, P., Ehrmann, M.A., Vogel, R.F. 2001. Monitoring the growth of *Lactobacillus* species during a rye bran fermentation. Food Microbiol. 18, 217–227.
- Muyzer, G., Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. A. van Leeuwenhoek, 73, 127–141.
- Naser, S.M., Thompson, F.L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M., Swings, J. 2005. Application of multi-locus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. Microbiology 151, 2141–50.
- Ning, W., Mackie, R.I., Gaskins, H.R. 1997. Biotype and ribotype diversity among *Lactobacillus* isolates from mouse ileum. Syst. Appl. Microbiol. 20, 423–31.
- Palys, T., Nakamura, L.K., Cohan, F.M. 1997. Discovery and classification of ecological diversity in the bacterial world: the role of DNA sequence data. Int. J. Syst. Bacteriol. 47, 1145–1156.
- Pavlovic, M., Hörmann, H., Vogel, R.F., Ehrmann, M.A. 2005. Transcriptional response reveals translation machinery as target for high pressure in *Lactobacillus sanfranciscensis* Arch. Microbiol. 26, 1–7.
- Pepe, O., Blaiotta, G., Anastasio, M., Moschetti, G., Ercolini, D., Villani, F. 2004. Technological and molecular diversity of *Lactobacillus plantarum* strains isolated from naturally fermented sourdoughs. Syst. Appl. Microbiol. 27, 443–53.
- Pulvirenti, A., Caggia, C., Restuccia, C., Gullo, M., and Giudici, P. 2001. DNA fingerprinting methods used for identification of yeasts isolated from Sicilian sourdough. Ann. Microbiol. 51, 107–120.
- Pulvirenti, A., Solieri, L., Gullo, M., De Vero, L., Giudici, P. 2004. Occurrence and dominance of yeast species in sourdough. Lett. Appl. Microbiol. 38, 113–7.
- Reinkemeier, M., Röcken, W. 1995. Einsatz definierter Starterkulturen zur Herstellung von Weizensauerteigbrot. - 1. Mitt.: Schnellmethode zur Isolierung von Plasmiden aus Starterkulturen der Gattung *Lactobacillus*. Getreide Mehl und Brot, 49, 93–98.
- Rodtong, S., Tannock, G.W. 1993. Differentiation of *Lactobacillus* strains by ribotyping. App. Environ. Microbiol., 59, 480–4.
- Roos, S., Lindgren, S., Jonsson, H. 1999. Autoaggregation of *Lactobacillus reuteri* is mediated by a putative DEAD-box helicase. Mol. Microbiol. 32, 427–436.
- Rossi, J. 1996. The yeasts in sourdough. Adv. Food Sci. 18, 201–211.
- Sakamoto, K., Margolles, A., van Veen, H.W., Konings, W.N. 2001. Hop resistance in the beer spoilage bacterium *Lactobacillus brevis* is mediated by the ATP-binding cassette multi-drug transporter HorA. J. Bacteriol. 183, 5371–5375.
- Salovaara, H., Savolainen, J. 1984. Yeast type isolated from sour rye dough starters. Acta Alim. Pol. 10, 241–245.
- Sarra, P.G., Pattarini, F., Bortolotti, F., Romani, A. 1992. Caratterizzazione di lattobacilli isolati di madri acide, Ind. Aliment. 31, 882–890.

- Satoh, E., Leer, R.J., Conway, P.L., Pouwels, P.H. 1999. In 6th FEMS symposium on lactic acid bacteria genetics, metabolism and applications, Mucus adhesion promoting protein of *Lactobacillus reuteri* 104R, Veldhoven, 19–23 September.
- Scheirlinck, I., Van der Meulen, R., Van Schoor, A., Cleenwerck, I., Huys, G., Vandamme, P., De Vuyst, L., Vancanneyt, M. 2007. *Lactobacillus namurensis* sp. nov., isolated from a traditional Belgian sourdough. *Int. J. Syst. Evol. Microbiol.* 57, 223–7.
- Scheyhing, C.H. 2003. Hochdruckinduzierte Genexpression bei Bakterien. PhD thesis, Technische Universität München, Munich, Germany.
- Schleifer, K.H., Ludwig, W. 1995a. Phylogeny of the genus *Lactobacillus* and related genera. *Syst. Appl. Microbiol.* 18, 461–467.
- Schleifer, K.H., Ludwig, W. 1995b. Phylogenetic relationships of lactic acid bacteria. In: Wood, B.J.B. Holzapfel W.H. (Eds), *The genera of lactic acid bacteria*. London: Chapman and Hall.
- Schleifer, K.H., Ehrmann, M., Beimfohr, C., Brockmann, E., Ludwig, W., Amann, A. 1995. Application of molecular methods for the classification and identification of lactic acid bacteria. *Int. Dairy J.* 5, 1081–1094.
- Settanni, L., Van Sinderen, D., Rossi, J., Corsetti, A. 2005. Rapid differentiation and *in situ* detection of 16 sourdough *Lactobacillus* species by multiplex PCR. *App. Environ. Microbiol.* 71, 3049–3059.
- Settanni, L., Valmorri, S., van Sinderen, D., Suzzi, G., Paparella, A., Corsetti, A. 2006. Combination of multiplex PCR and PCR-denaturing gradient gel electrophoresis for monitoring common sourdough-associated *Lactobacillus* species. *App. Environ. Microbiol.* 72, 3793–6.
- Siranganathan, N., Seidler, R.J., Sandine, W.E. 1985. Nucleic acids of species of *Lactobacillus*. *J. Dairy Sci.* 68, 1077–1086.
- Spicher, G. 1959. Die Mikroflora des Sauerteiges. I. Mitteilung: Untersuchungen über die Art der in Sauerteigen anzutreffenden stäbchenförmigen Milchsäurebakterien (Genus *Lactobacillus* Beijerinck), *Zeitblatt für Bakteriologie II Abt* 113, 80–106.
- Spicher, G. 1984. Weitere Untersuchungen über die Zusammensetzung und die Variabilität der Mikroflora handelsüblicher Sauerteig-Starter, *Zeitschr. Lebensm. Unters. Forsch.* 178, 106–109.
- Spicher, G. 1987. Die Mikroflora des Sauerteiges. XXII. Mitteilung: Die in Weizensauerteigen vorkommenden *Lactobacillen*, *Zeitschr. Lebensm. Unters. Forsch.* 184, 300–303.
- Spicher, G., Schröder, R. 1980. Die Mikroflora des Sauerteiges. VIII. Die Faktoren des Wachstums der im ‘Reinzuchtsauer’ auftretenden Hefen. *Z. Lebensm. Unters. Forsch.* 170, 119–123.
- Spicher, G., Schröder, R. 1978. Die Mikroflora des Sauerteiges. IV. Mitteilung: Untersuchungen über die Art der in ‘Reinzuchtsauern’ anzutreffenden stäbchenförmigen Milchsäurebakterien (Genus *Lactobacillus* Beijerinck), *Zeitschr. Lebensm. Unters. Forsch.* 167, 342–354.
- Stackebrandt, E., Goebel, B.M. 1994. Taxonomic note: a place for DNA-DNA re-association and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44, 846–849.
- Stahl, M., Molin, G. 1994. Classification of *Lactobacillus reuteri* by restriction endonuclease analysis of chromosomal DNA. *Int. J. Syst. Bacteriol.* 44, 9–14.
- Steudel, U.L. 2001. Physiologische und molekulare Charakterisierung der Stressantwort von *Lactobacillus pontis* und *Lactobacillus sanfranciscensis*. PhD thesis, Technische Universität München, Munich, Germany.
- Stiles, M.E., Holzapfel, W.H. 1997. Lactic acid bacteria of foods and their current taxonomy. *Int. J. Food. Microbiol.*, 36, 1–29.
- Strohmar, W., Diekmann, H. 1992. Die Mikrobiologie eines Langzeit-Sauerteiges. *Zeitschr. Lebensmitteluntersuchung Forsch.* 194, 536–540.
- Sugihara, T.F., Kline, L., Miller, M.W. 1971. Microorganisms of the San Francisco sourdough bread process. I. Yeasts responsible for the leavening action. *Appl. Microbiol.* 21, 456–458.
- Suihko, M.L., Mäkinen, V. 1984. Tolerance of acetate, propionate and sorbate by *Saccharomyces cerevisiae* and *Torulopsis holmi* (yeasts in bakery fermentation). *Food Microbiol.* 1, 110–115.

- Tieking, M., Ganzle, M.G. 2005. Exopolysaccharides from cereal associated lactobacilli. *Trends Food Sci. Technol.* 16, 79–84.
- Tieking, M., Ehrmann, M.A., Vogel, R.F., Ganzle, M.G. 2005. Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Appl. Microbiol. Biotechnol.* 66, 655–63.
- Tieking, M., Korakli, M., Ehrmann, M.A., Ganzle, M.G., Vogel, R.F. 2003. *In situ* production of exopolysaccharides during Sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *App. Environ. Microbiol.* 69, 945–52.
- Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K. 2001. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. *Syst. Appl. Microbiol.* 24, 554–60.
- Turner, M.S., Timms, P., Hafner, L.M., Giffard, P.M. 1997. Identification and characterization of a basic cell surface-located protein from *Lactobacillus fermentum* BR11. *J. Bacteriol.* 179, 3310–3316.
- Turner, M.S., Woodberry, T., Hafner, L.M., Giffard, P.M. 1999. The *bspA* locus of *Lactobacillus fermentum* BR11 encodes a L-cysteine uptake system. *J. Bacteriol.* 181, 2192–2198.
- Urwin, R., Maiden, M.C.J. 2003. Multi-locus sequence typing: a tool for global epidemiology. *Trends Microbiol.* 11, 479–487.
- Valcheva, R., Ferchichi, M.F., Korakli, M., Ivanova, I., Ganzle, M.G., Vogel, R.F., Prevost, H., Onno, B., Dousset, X. 2006. *Lactobacillus nantensis* sp. nov., isolated from French wheat sourdough. *Int. J. Syst. Evol. Microbiol.* 56, 587–91.
- Valcheva, R., Korakli, M., Onno, B., Prevost, H., Ivanova, I., Ehrmann, M.A., Dousset, X., Ganzle, M.G., Vogel, R.F. 2005. *Lactobacillus hammesii* sp. nov., isolated from French sourdough. *Int. J. Syst. Evol. Microbiol.* 55, 763–7.
- Valsangiacomo, C., Baggi, F., Gaia, V., Balmelli, T., Peduzzi, R., Piffaretti, J.C. 1995. Use of amplified fragment length polymorphism in molecular typing of *Legionella pneumophila* and application to epidemiological studies. *J. Clin. Microbiol.* 33, 1716–1719.
- Van Hijum, S.A., van Geel-Schutten, G.H., Rahaoui, H., van der Maarel, M.J., Dijkhuizen, L. 2002. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *App. Environ. Microbiol.* 68, 4390–4398.
- Vancanneyt, M., Neysens, P., De Wachter, M., Engelbeen, K., Snauwaert, C., Cleenwerck, I., Van der Meulen, R., Hoste, B., Tsakalidou, E., De Vuyst, L., Swings, J. 2005. *Lactobacillus acidifarinae* sp. nov. and *Lactobacillus zymae* sp. nov., from wheat sourdoughs. *Int. J. Syst. Evol. Microbiol.* 55, 615–20.
- Ventura, M., Canchaya, C., Del Casale, A., Dellaglio, F., Neviani, E., Fitzgerald, G.F., van Sinderen, D. 2006. Analysis of bifidobacterial evolution using a multi-locus approach. *Int. J. Syst. Evol. Microbiol.* 56, 2783–92.
- Vermeulen, N., Pavlović, M., Ehrmann, M.A., Vogel, R.F., Gänzle, M.G. 2005. Functional characterization of the proteolytic system of *L. sanfranciscensis* TMW 1.53 during growth in sourdough. *App. Environ. Microbiol.* 71, 6260 – 6266.
- Vermeulen, N., Thiele, C., Gänzle, M.G. Vogel, R.F. 2003. Cysteine metabolism by cereal associated lactobacilli. In *Second International Symposium on Sourdough, From Fundamentals to Applications*, Brussels, 8–11 October 2003.
- Vogel, R.F., Böcker, G., Stolz, P., Ehrmann, M., Fanta, D., Ludwig, W., Pot, B., Kersters, K., Schleifer, K.H., Hammes, W.P. 1994. Identification of Lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. *Int. J. Syst. Bacteriol.* 44, 223–229.
- Vogel, R.F., Ehrmann, M.A., Gänzle, M.G. 2002. Development and potential of starter lactobacilli resulting from exploration of the sourdough ecosystem. *A. van Leeuwenhoek*, 81, 631–638.
- Vogel, R.F., Knorr, R., Müller, R.A., Steudel, U., Gänzle, M.G., Ehrmann, M.A. 1999. Non-dairy lactic fermentations: the cereal world. *A. van Leeuwenhoek* 76, 403–411.
- Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T. Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. 1995. AFLP – a new technique for DNA fingerprinting. *Nucl. Acids Res.* 23, 4407–4414.

- Walter, J., Heng, N.C., Hammes, W.P., Loach, D.M., Tannock, G.W., Hertel, C. 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. *App. Environ. Microbiol.*, 69, 2044–2051.
- Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E., Starr, M.P., Trüper, H.G. 1987. Report of the Ad Hoc Committee on Reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37, 463–464.
- Welsh, J.McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nuc. Acids Res.* 18, 7213–7218.
- Wiese, B.G., Strohmar, W., Rainey, F.A., Diekmann, H. 1996. *Lactobacillus panis* sp. nov., from Sourdough with a Long fermentation period. *Int. J. Syst. Bacteriol.* 46, 449–453.
- Yagasaki, M., Iwata, K., Ishino, S., Azuma, M., Ozaki, A. 1995. Cloning, purification, and properties of a cofactor-independent glutamate racemase from *Lactobacillus brevis* ATCC 8287. *Biosci. Biotechnol. Biochemi.* 59, 610–614.
- Zapparoli, G., Torriani S. 1997. Rapid identification and detection of *Lactobacillus sanfrancisco* in sourdough by species-specific PCR with 16S rRNA-targeted primers. *Syst. Appl. Microbiol.* 20, 640–644.
- Zapparoli, G., Toriani, S., Dellaglio, F. 1998. Differentiation of *Lactobacillus sanfranciscensis* strains by randomly amplified polymorphic DNA and pulsed field gel electrophoresis. *FEMS Microbiol. Lett.* 166, 325–332.
- Zhong, W., Millsap, K., Bialkowska-Hobrzanska, H., Reid, G. 1998. Differentiation of *Lactobacillus* species by molecular typing. *App. Environ. Microbiol.*, 64, 2418–23.

Chapter 5

Vegetable Fermentations

**Hikmate Abriouel, Nabil Ben Omar, Rubén Pérez Pulido,
Rosario Lucas López, Elena Ortega, Magdalena Martínez Cañamero,
and Antonio Gálvez**

Abstract Many different vegetable fermentation processes are currently carried out on an industrial scale, most of which still rely on selection of the autochthonous microbiota of the raw materials and fermentation plant. The implication of lactic acid bacteria in such processes has been deciphered by classical microbiological techniques in most cases. The application of DNA-based culture-dependent and culture-independent analyses may provide new insights into the microbial successions that take place during the fermentation as well as the microbial diversity. These data can be linked to other issues such as flavor development or regional differences in fermented foods. DNA-based methods can also help to evaluate the fitness of starter cultures used for vegetable fermentations. In this chapter, advances in the understanding of several vegetable fermentations (including caper berries, “Almagro” eggplants, sauerkraut and table olives) by use of molecular techniques are discussed.

1 Introduction

Fermentation is one of the oldest methods of food preservation technology in the world. The process relies on the biological activity of microorganisms for production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs. As a result, fermented products generally have a longer shelf life than their original substrate and present very good safety records. Thanks to this, fermentation has enabled our ancestors in the tropics to survive drought periods and those in cooler regions to survive winter seasons.

As a technology, food fermentation dates back at least 8,000 years. It is thought that in this time period the art of cheese making was developed in the fertile Crescent between the Tigris and Euphrates Rivers in Iraq, at a time when plants and animals were just being domesticated (Fox 1993). Fermentation of milk started in many places with evidence of fermented products in use in Babylon over 5,000 years ago, and there is also evidence of fermented meat products being produced for King Nebuchadnezer of Babylon. Bread making probably originated in Egypt over 3,500 years ago (Sugihara 1985). Alcoholic fermentations involved in winemaking and

brewing are thought to have developed during the period 2,000 to 4,000 BC by the Egyptians and Sumerians. Through the ages, fermentation has had a major impact on nutritional habits and traditions, on culture and on the commercial distribution and storage of food.

The principal substrates used for fermented products are milk, meat and vegetables. These generate over 400 varieties of cheese (Jay, et al. 2005) and a very ample range of yogurt and fermented milk drinks, fermented Sausages as well as fermented vegetables. The increasing interest in fermented vegetable products in several European countries is the main reason for starting new research in this field. In Europe, there are over 21 different commercial vegetable fermentations and the most economically relevant of these are the fermentations of olives, cucumbers, and cabbage (Bückerhuskes 1997). The size of the European industry is significant. More than half of the olives and cucumbers are produced in Spain, while nearly 50 percent of the sauerkraut is fermented in Germany. In addition to this, a large number of fermented vegetable foods are also made on a small scale or at home by using traditional methods. These deserve a special recognition, not only for the unique flavor and taste of the final products, but also for their contribution to preservation of cultural heritage and biodiversity.

As raw vegetables have a high microbial load and cannot be pasteurized without compromising product quality, most vegetable fermentations occur as a consequence of providing growth conditions (such as added salt) that favour the lactic acid bacteria (LAB). These bacteria are present on fresh vegetables in very low numbers, accounting for only 0.15 percent to 1.5 percent of the total population (Bückerhuskes 1997). Earlier studies of the microbial communities of fermented vegetables have serious limitations. First, the organisms present were isolated by conventional culture methods using limited types of media and culture conditions, and it is now well known that bacteria in nature cannot all be cultured under general laboratory conditions. Second, species were identified by phenotypic methods, especially by biochemical analysis such as sugar fermentation patterns and by cell morphology. This inevitably leads to mis-identification and misinterpretation in microbial community studies. In recent years the advent of molecular biology techniques is throwing new light on the microbial ecology of traditional fermented vegetables, opening a new window (and probably also a new era) in food fermentation. This chapter addresses a few examples in which application of molecular techniques has significantly contributed to our improved knowledge of the microbial diversity of food ecosystems.

2 Fermentation of Caper Berries

Caper berries are the fruits of *Capparis* species (mainly *Capparis spinosa* L.), a Mediterranean shrub that grows wild in semi-arid regions and is also cultivated for its buds and fruits. The caper berry resembles a large grape with white stripes, or actually more like a teeny watermelon. Fermented capers are often served as an

appetizer with meat, olives, cheese, and nuts, or as a complement to salads, pasta, and other foods. The main producers of fermented caper berries are Mediterranean countries, especially Greece, Italy, Turkey, Morocco and Spain, and the final products are exported mainly to central European countries, the United States, and the United Kingdom as a delicatessen product. Traditional fermented capers are highly appreciated for their unique organoleptic properties.

Fermentation of caper fruits is often done by traditional artisanal ways (Luna and Perez 1985). The fruits are collected during the months of June and July and immersed in tap water. Fermentation vessels are placed on sunny terraces where the fermentation takes place for approximately five to seven days in a temperature ranging from 20 to 45 °C. Fermented capers are then placed in brine and distributed for consumption. Production of fermented caper fruits, like other natural vegetable fermentation, is a spontaneous lactic acid fermentation based on an empirical process which relies upon microorganisms present in the raw material and processing environment (Alvaruiz, et al. 1990; Özcan 1999, 2001; Özcan and Akgül 1999a, b).

The microbial communities and population dynamics of caper fermentation were described recently by using a combination of classical and molecular techniques (Pérez Pulido, et al. 2005). The results obtained following conventional methods indicate that LAB dominate this fermentation. Following molecular identification methods using species-specific PCR primers and 16S rRNA gene sequencing, the following species were identified: *Lactobacillus plantarum*, *Lb. paraplantarum*, *Lb. pentosus*, *Lb. brevis*, *Lb. fermentum*, *Pediococcus pentosaceus*, *P. acidilactici*, and *Enterococcus faecium* (Pérez Pulido, et al. 2005). *Lb. plantarum* was the predominant LAB in caper fermentation (representing ca. 49 percent of isolates), and it was detected during the whole fermentation period (Fig. 5.1). *Lb. brevis* and *Lb. pentosus* were detected intermittently (probably as a result of their lower abundance), while *Lb. fermentum* was detected towards the end of the fermentation (days 5 to 7). The pediococci clearly predominated at the early stages of fermentation and then were overcome by lactobacilli (Fig. 5.1).

The population dynamics of caper fermentation were also studied by using a culture-independent approach based on temporal temperature gel electrophoresis (TTGE) analysis. In this procedure, DNA was amplified by nested PCR, separated by TTGE, and gels were visually inspected to identify the bands representing the populations involved in the fermentation (Fig. 5.2). To circumvent the biases inherent in subjective interpretation, the identification of bacteria was confirmed by direct sequencing of DNA bands. When the results obtained from both traditional plating and TTGE were analyzed, it became evident that the fermentation was characterized by strong LAB activity. The profiles obtained by TTGE allowed the identification of the main groups of LAB (*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Pediococcus* sp., *Enterococcus* sp., *E. faecium*, and *E. casseliflavus*). In agreement with results obtained by culture-dependent methods, these results also show that the predominant species in this fermentation process is *Lb. plantarum*, which was represented by a highly intense band throughout the fermentation. The remaining bacterial groups yielded bands of lower intensities by TTGE, suggesting a lower abundance, with slight variations along the fermentation. Greatest differences

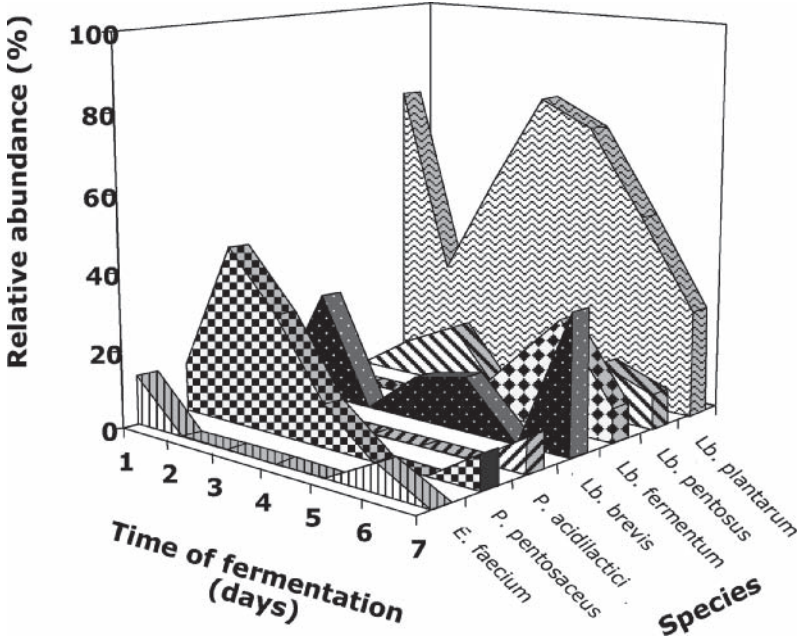


Fig. 5.1 Relative abundance of the different LAB during the course of time in caper fermentation (reprinted from Pérez Pulido, et al. 2005, with permission)

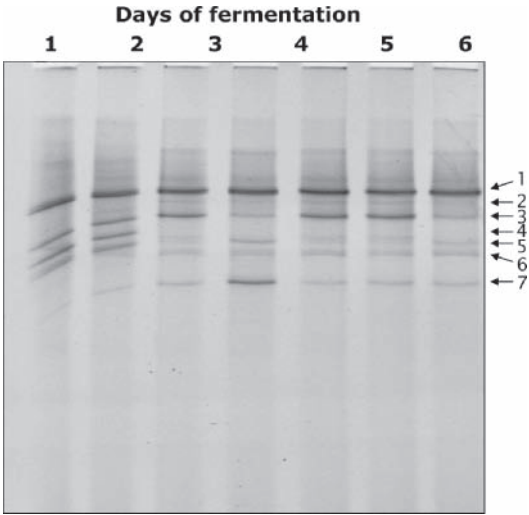


Fig. 5.2 Temporal temperature gradient gel electrophoresis of PCR-amplified 16S DNA from samples taken at different times during caper fermentation (lanes 1 to 7). DNAs from the different bands were extracted, sequenced, and compared with a database for identification: 1, *Lactobacillus plantarum*; 2, *Enterococcus casseliflavus*; 3, *Lb. fermentum*; 4, *Enterococcus* sp.; 5, *Lb. brevis*; 6, *E. faecium*; 7, *Pediococcus* sp. (reprinted from Pérez Pulido, et al. 2005, with permission)

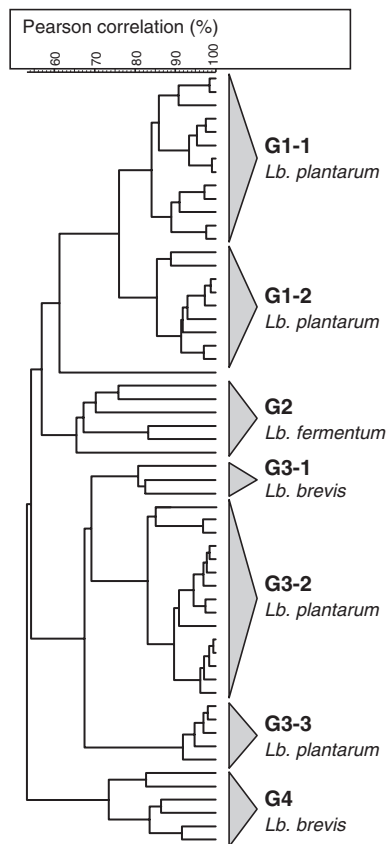
were noticed for *Enterococcus* sp. and *Lb. brevis* bands, which were clearly more intense during days 1 and 2 (Fig. 5.2).

Comparison of results from molecular and culture-dependent studies revealed similar species composition for caper fermentation, and all species of *Lactobacillus* were correctly identified in both cases. However, *Pediococcus* could only be identified at species level by culture-dependent methods, while TTGE provided a more accurate picture of the distribution of this bacterium through the fermentation. *E. faecium* was also identified in both cases, but once more, its distribution during the fermentation could only be determined by TTGE. Another enterococcal species (*E. casseliflavus*) could only be detected by TTGE analysis. These results suggest that TTGE analysis can offer better results for detection of species that are present at lower concentrations. However, it should also be considered that amplification results may largely be dependent on the extraction efficacy for different bacterial species and food matrices. Results from other works also revealed significant differences in the microbial compositions of fermented foods depending on the use of culture-dependent or culture-independent methods (Ben Omar and Ampe 2000; Ercolini, et al. 2001; Meroth, et al. 2003; Miambi, et al. 2003), suggesting that polyphasic studies should be used to better understand the microbial ecology of foods.

Caper fruit fermentation shares many characteristics with other vegetable fermentations, where *Lb. plantarum* is usually the predominant species as well, both because of its higher acid tolerance and its ability to degrade sugars which are present in vegetables (Daeschel, et al. 1987; Ruiz-Barba, et al. 1994; Garrido Fernández, et al. 1995; Leal-Sánchez, et al. 2003). However, while *Leuconostoc* species are also present in other vegetable fermentations, they were not detected in capers. The absence of *Leuconostoc* could be attributed to the rapid decrease of pH, since this bacterium is not able to grow below pH 4.5 (Montaño, et al. 1992). Moreover, it should be noted that this process takes place during a very warm time of the year, and the exposure of the containers to the solar radiation allows the fermentation to take place at temperatures over 40°C, and growth of *Leuconostoc* seems to be favored in fermentations that take place at low temperatures, between 8 and 18°C (Pederson, et al. 1954). Therefore, the high temperatures in caper fermentations could inhibit *Leuconostoc* growth.

The biodiversity of LAB from caper fermentation was studied by randomly amplified polymorphic DNA (RAPD)-PCR fingerprinting (Pérez Pulido, et al. 2005). Cluster analysis of RAPD-PCR patterns revealed a high degree of diversity among lactobacilli, which could be separated into four different groups (Fig. 5.3): G1 (*Lb. plantarum*, *Lb. pentosus*), G2 (*Lb. fermentum*), G3 (*Lb. brevis*, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. pentosus*) and G4 (*Lb. brevis*). Groups G1 and G3 were the most diverse, and branched in two and three subgroups, respectively. However, according to biochemical tests, the lactobacilli were more homogeneous and most strains of *Lb. plantarum* clustered in a single group (Pérez Pulido, et al. 2007). The pediococci isolated from caper fermentation represented a much more homogeneous group as far as RAPD-PCR patterns (Pérez Pulido, et al. 2005) and biochemical profiles, although they could be separated in different groups according to plasmid content (Pérez Pulido, et al. 2006).

Fig. 5.3 Dendrogram obtained after RAPD-PCR analysis of rod-shaped LAB isolated from caper fermentation (adapted from Pérez Pulido, et al. 2005, with permission)



The results of culture-dependent and molecular studies altogether reveal the microbial complexity of caper berry fermentation. This would be expected for a natural fermentation, which relies solely on the microbiota of the fruit surface, manufacturing plant and other natural sources. Fermented capers were also shown to be a valuable source of LAB strains with functional properties, including the capacity to degrade raffinose and stachyose, as well as other properties of interest such as phytase and bile salt hydrolase activities (Pérez Pulido, et al. 2007). Therefore, this traditional fermentation process may be a valuable source of new strains with potential industrial applications.

3 Fermentation of “Almagro” Eggplants

“Almagro” eggplants are elaborated almost exclusively in the region surrounding the town of Almagro, in the province of Ciudad Real (Spain), and are widely consumed as an appetizer; they are also used to prepare other culinary dishes. They are

manufactured using the native “Almagro” variety of eggplant, *Solanum melongena* L. var. *esculetum depressum*. The fruits are processed, blanched and placed as whole pieces in plastic containers (with a capacity of around 250 l) with brine. The brines may contain sodium chloride (3.8 percent), vinegar (to a final concentration of 6 percent w/v acetic acid in the brine), citric acid (0.07 percent) as well as condiments and spices (garlic powder, cumin, paprika, and saffron; Seseña, et al. 2001). “Almagro” eggplants are, therefore, a pickled product with an initial pH that may be as low as 2.7. Spontaneous fermentation takes place at ambient temperature for about seven days. The manufacture of this pickle has been controlled by a “Berenjena de Almagro” Denomination of Origin since 1994 (Sánchez, et al. 2000). Manufacture of this product is strictly seasonal, from a single annual crop harvested from June to September, although recent developments have enabled frozen eggplants to be used to manufacture this product (Palop and Ballesteros 2000). This has prompted investigation of the microbiology of eggplant fermentation and the development of appropriate starter cultures that will allow the manufacture of “Almagro” eggplants outside the normal growing season.

The microbiological aspects of “Almagro” eggplant fermentation have been elucidated in the last decade. Spontaneous fermentation of “Almagro” eggplants is homogeneous as far as the microbial genera involved, with the almost exclusive predominance of lactobacilli (Sánchez, et al. 2000). This is a main difference from other fermentations such as olives, sauerkraut or cucumbers (in which species of the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc* commonly occur) as well as capers (Daeschel, et al. 1987; Montaña, et al. 1992; Pérez Pulido, et al. 2005). The absence of *Pediococcus* and *Leuconostoc* has been attributed to inhibition caused by the low initial pH of the brine used in this fermentation process (ca. 2.7), which is much lower than the minimum pH values (4.0 and 4.5, respectively) tolerated by these species (Montaña, et al. 1992). Similarly to caper fermentation, the absence of *Leuconostoc* could also be explained by the relatively high temperatures at which this fermentation takes place, as it is carried out during the warm season of the year.

Spontaneous “Almagro” eggplant fermentation is initiated by the obligate heterofermentative species *Lb. fermentum*/*Lb. cellobiosus* and *Lb. brevis* (Sánchez, et al. 2000; Seseña, et al. 2004). During the fermentation, the facultative heterofermentative species *Lb. plantarum* (as well as *Lb. pentosus*) becomes predominant (Sánchez, et al. 2000; Seseña, et al. 2004), similarly to other fermented vegetables (Fleming 1982; Daeschel, et al. 1987; Oyewole and Odunfa 1990; Tamang and Sarkar 1996; Kunene, et al. 2000; Mugula, et al. 2003; Pérez Pulido, et al. 2005). The sensory attributes of this pickled product derive mainly from the contribution of *Lb. plantarum*, and to a lesser extent from obligate heterofermentative lactobacilli producing acetic acid, carbon dioxide, and ethanol, as well as lactic acid (Kandler 1983; Hounhouigan, et al. 1993; Seseña, et al. 2001).

A study carried out on the biochemical and physiological characterization of lactobacilli obtained from spontaneous eggplant fermentations revealed six groups (Sánchez, et al. 2000), which were identified as *Lb. plantarum* biotype 1 (54.4 percent), *Lb. brevis* biotype 2 (19.5 percent), *Lb. fermentum* (9.4 percent), *Lb. brevis* biotype 3 (5.4 percent), *Lb. pentosus* (4.7 percent), and nine strains grouped as *Lactobacillus* spp. (6.0 percent). Biotype profiles showed good correlation with

SDS-PAGE whole cell protein fingerprints (Sánchez, et al. 2003). Further discrimination was achieved when combined numerical analysis of the results from SDS-PAGE whole cell protein analysis together with RAPD-PCR and pulsed-field gel electrophoresis (PFGE) was carried out, indicating a considerable degree of genomic diversity in the LAB microbiota studied, and especially in the *Lb. plantarum* isolates (Sánchez, et al. 2004).

The study of genetic diversity in LAB from industrial fermentation of “Almagro” eggplants also revealed large differences from one enterprise to another (Seseña 2005). Among 127 genotypes detected by RAPD analysis in three enterprises sampled, only three of them belonged to the same strain, suggesting that only a very low percentage of strains (1.9 percent) from this fermentation are cosmopolitan, in spite of the geographic proximity of the analyzed enterprises.

Differences in genetic diversity of isolates from eggplant fermentation were also reported between two different seasons, as well as regarding the species represented (Seseña, et al. 2004). Comparative analysis of isolates from two seasons revealed 34 distinct RAPD patterns with 95 percent of isolates grouped into 18 main clusters. While the predominant species for season I was *Lb. plantarum* (58.0 percent), *Lb. fermentum*/*Lb. cellobiosus* predominated in season II (88.5 percent). Brines from season II showed a higher genetic diversity, and the greatest numbers of genotypes were found among *Lb. plantarum* and *Lb. fermentum*/*Lb. cellobiosus* species. Several isolates from both seasons showed the same *Lb. fermentum*/*Lb. cellobiosus* genotype, suggesting they were endemic to that factory (Seseña, et al. 2004). The degree of genetic diversity seems not to be essential for the production of fermented “Almagro” eggplants, but may cause variations in organoleptic characteristics of manufactured products between different producing units and seasons as well. Therefore, the use of starter cultures could be recommended to improve product homogeneity.

Addition of starter cultures may accelerate the manufacturing process and avoid problems associated with uncontrolled fermentations, such as variations in product quality and taste, and at the same time allow the use of brines with lower salt or acid content (Seseña, et al. 2001). Inoculating “Almagro” eggplants with a commercial vegetable starter culture showed an acceleration of the fermentation process and shortened the fermentation time, provided that the salt concentration in the brine did not exceed 6 percent (Ballesteros, et al. 1999). However, the fermented product had an atypical and unacceptable bitter taste, probably due to the inadequacy of the starter culture used. To avoid this problem different studies were carried out using selected LAB isolated from natural eggplant fermentations. Seseña, et al. (2001) showed that the most appropriate starter for the manufacture of fermented “Almagro” eggplants should contain the obligate heterofermentative species *Lb. brevis* and the facultative heterofermentative species *Lb. plantarum*. The selected mixed starter improved the sensory properties of the fermented product, compared to commercial samples, and shortened the fermentation time.

Freeze-storage of eggplants before fermentation would allow an extension of the manufacturing season as well as long-distance transportation of eggplants from other geographical regions. Although frozen eggplants will yield a finished product of acceptable quality, the freezing step causes a delay on the onset of fermentation

(Seseña, et al. 2002); therefore the use of starter cultures would be recommended to solve this problem. Selected strains of *Lb. plantarum*, *Lb. fermentum* and *Lb. brevis* isolated from spontaneous fermentations were tested as starter cultures alone and/or in combination, and their predominance in the fermentation of frozen eggplants was studied by RAPD-PCR (Seseña, et al. 2005). When used as a starter culture alone, *Lb. plantarum* was found to predominate in the fermentation of frozen eggplants. However, when used in combination with *Lb. pentosus* or *Lb. pentosus/Lb. brevis*, *Lb. pentosus* was always the predominant LAB, with increased growth of *Lb. brevis* at the end of fermentation (Seseña, et al. 2005). Although the course of the fermentation was similar in all cases, sensorial analysis provided best results for eggplants fermented with a mixture of the three strains. Results of fermentation for frozen eggplants did not differ from commercial eggplants, strengthening the adequacy of starters for fermentation of the frozen fruits.

4 Other Fermented Vegetables

4.1 Sauerkraut

Sauerkraut fermentation is carried out with dry salting. Cabbage leaves are washed in potable cold water, drained and placed in layers about 2.5 cm deep in fermenting containers of 45 to 150 tons (Fleming, et al. 1988). Salt is sprinkled over all and then another layer of vegetables is added, followed by more salt. This is repeated until the container is three-quarters full. Salt extracts the juice from the vegetable tissues, thus forming the brine. A cloth is placed above the vegetables and a weight added to compress the vegetables and facilitate brine formation and expulsion of air. Oxygen is rapidly consumed by plant cells and aerobic microbiota, creating anaerobic conditions. Fermentation starts right after formation of the brine, and the process lasts between one to four weeks, depending on the ambient temperature.

Commercial sauerkraut fermentation is generally initiated by the natural LAB microbiota present on the cabbage. Typically, four LAB species are represented: *Leuconostoc mesenteroides*, *P. pentosaceus*, *Lb. brevis*, and *Lb. plantarum*. The major species involved in the early stage of sauerkraut fermentation is *Lc. mesenteroides*, while *Lb. plantarum* becomes predominant at a later stage, at days 5 to 7. The correct sequence of organisms, and especially the early predominance of heterofermentative LAB, are essential in achieving a stable product with flavor and aroma typical of sauerkraut (Pederson and Albury 1969). Therefore, the introduction of *Lc. mesenteroides* starter cultures could help ensure the desired fermentation (Fleming, et al. 1995). To determine the ability of starter cultures to predominate over the naturally present microflora, a method for identifying individual strains of *Lc. mesenteroides* is needed. However, classification of individual strains is difficult since *Lc. mesenteroides* strains have very similar physiological properties and nutritional requirements. Plengvidhya, et al. (2004) used RAPD-PCR to determine

the growth, survival, and predominance of *Lc. mesenteroides* starter cultures in sauerkraut fermentation. Nine strains of *Lc. mesenteroides* used as starters were distinguished from each other by their RAPD profiles. A pilot scale fermentation was inoculated with unmarked and marked starter cultures, and isolates from spiral plates were collected. The similarity of RAPD profiles among isolates and starter cultures was determined as using the Fuzzy logic coefficient and NJ clustering method. RFLP analysis of the rRNA gene-ITS region was also used to confirm RAPD-PCR identification for differentiating starter cultures and isolates. The patterns observed from the isolates taken at two and five days of fermentation were identical to the starter. While some variation in the RAPD patterns was observed, the results showed that the starter cultures dominated the fermentation during the early heterofermentative stage. The selected isolates were confirmed as the added starter cultures when typed by PFGE. These results demonstrated the utility of RAPD to follow the progression of unmarked starter cultures of *Lc. mesenteroides* in sauerkraut fermentations, providing a new tool for a more precise study of this vegetable fermentation at molecular levels.

4.2 Table Olives

The production of fermented olives for human consumption is a tradition of the Mediterranean region that has now extended to other regions of the world (Leone 2000). The unripened (or green olives) as well as the ripened fruits (or black olives) are processed either under natural conditions or at industrial scale using more complex methods to derive a variety of fermented products. In the most popular Spanish-style green table olive processing method, green olives are treated with a sodium hydroxide (lye) solution (1.8 percent to 2.5 percent) to eliminate the fruit's main bitter glucoside compound (oleuropein). After the glucoside has been removed, the olives are placed in barrels with a brine solution (10 percent to 13 percent NaCl) and allowed to undergo a lactic fermentation (Garrido Fernández, et al. 1997). The optimal fermentation temperature is 24 °C. The fermentation period usually takes two to three months. Once fermentation is complete, the olives are packed in airtight jars and sterilized. The finished product has good quality characteristics with a long storage life. Indigenous LAB change spontaneously during natural fermentation and the *Lactobacillus* species predominate at the end of the process, mainly *Lb. plantarum* (Garrido Fernández, et al. 1997). However, physico-chemical characteristics (e.g., NaCl concentration, external temperature, etc.) are responsible for changes in the microbial flora during the fermentation. Yeasts are usually present during the fermentation process and storage, as well (Ruiz Barba, et al. 1994; Leal-Sánchez, et al. 2003). In some cases, when lactic acid is not produced in sufficient amounts for adequate preservation, microbial spoilage may occur (Fernández Díaz 1983; Garrido Fernández, et al. 1995). Interest in developing effective starter cultures to be used in fermentation of table olives is increasing, since industrial experience suggests that an appropriate inoculation reduces the

probability of spoilage and helps to achieve an improved and more predictable fermentation process. Research has been done on specific traits of interest, such as oleuropein-splitting capability (Servili, et al. 2006), bacteriocin production (Jiménez-Díaz, et al. 1993; Ruiz Barba, et al. 1994; Leal-Sánchez, et al. 2003; Maldonado, et al. 2003, 2004), growth in ripe olive brines (Durán, et al. 1993), and fermentation at low temperature (Durán Quintana, et al. 1999).

Homemade production of green table olives is a widespread practice in Mediterranean rural areas. In this process olives are usually placed in tap water for several days, and water may be replaced periodically as a way to eliminate hydro-soluble oleuropein. This process is more respectful of fruit microbiota than the commercial lye treatment. After washing steps, olives are placed in 5 percent to 10 percent NaCl brine and allowed to ferment for at least three to six months. Garlic and aromatic herbs may also be added, either during the fermentation or at the end of the fermentation period. The added NaCl and the progressive pH decrease select for a lactic acid microbiota that increases from 1 percent of the total microbial population in the fresh brine to over 80 percent after a few days (Robinson 1988).

During natural fermentation of green olives indigenous LAB change spontaneously, and *Lactobacillus* species, mainly *Lb. plantarum*, are believed to predominate at the end of fermentation (Fernández González, et al. 1993; Harris 1998). However, recent studies carried out on naturally fermented Sicilian green olives, based on restriction fragment length polymorphisms (RFLP)-PCR of 16S rRNA gene, showed a remarkable bacterial heterogeneity, and 16S rRNA gene sequencing revealed a strong domination of isolates belonging to *Lb. casei* (Randazzo, et al. 2004). Enterococcal species, including *E. casseliflavus*, *E. faecium* and *E. hirae*, were also identified. Another recent study investigated the presence of *Lb. plantarum*, *Lb. paraplantarum* and *Lb. pentosus* in 25 samples of green olives (cultivar “Leccino”) collected in the Campania region (Southern Italy) by fluorescent *in situ* hybridization (FISH) with species-specific probes (Ercolini, et al. 2006). However, none of the above-mentioned species of this genus were found in the raw material. Instead, investigation of the identity of colonies grown on Rogosa agar plates by PCR-DGGE revealed *Ln. pseudomesenteroides* to be the most frequently found species in the olive fruits. Other genera (*Pediococcus*, *Pseudomonas* and *Raoultella*) were also identified. These results suggest that the lactobacilli that drive the fermentation of table olives may originate from other sources such as the processing plant environment and fermentation tanks, as well as other tools used for production. Moreover, the use of FISH with species-specific probes seems an attractive approach to study not only the identity, but also the associations of LAB on the surface of table olives and in the fermentation brine. Additional molecular studies are needed to elucidate the LAB microbiota of green olives from different geographic regions, as well as the microbiological profiles of olives fermented by different methods.

Greek-style naturally black olives are made from ripened fruits, and have a distinctive softness and flavor. Ripened olives are washed and placed in 6 percent to 8 percent salt brine where they are left to ferment for several months (Garrido Fernández, et al. 1997). In addition to LAB, yeasts are largely responsible for this

fermentation process. Black olive fermentations have been studied in different countries – mainly Spain, Portugal, Greece and Morocco – and showed some yeast species biodiversity, with a few dominant species: *Pichia membranifaciens*, *Saccharomyces oleaginosus*, *P. anomala*, *Candida boidinii* and *Torulaspora delbrueckii* (Marquina, et al. 1992; Fernández González, et al. 1992; Kotzekidou 1997; Marquina, et al. 1997). By using rRNA gene ITS-PCR RFLP, a total of 11 different profiles were detected for yeasts isolated from black olive fermentation. According to RFLP profiles and/or sequencing of the D1/D2 region of the 26S rRNA gene, the following species could be identified: *P. anomala*, *P. membranifaciens*, *Debaryomyces hansenii*, *D. etchellsii*, *C. atlantica*, *C. boidinii*, *C. pararugosa* and *Zygoascus hellenicus* (Coton, et al. 2006). Results from this study concluded that analysis of ITS-PCR RFLP profiles could provide valuable information on yeast population biodiversity and dynamics in the naturally fermented black olives.

The balance between yeasts and LAB in black olive fermentation seems to be largely influenced by the brine salt concentration. Tassou, et al. (2002) reported that brines containing 4 percent or 6 percent NaCl favored a lactic fermentation (with high acidity levels and lower pH), while 8 percent NaCl brines affected the growth of LAB and favored the activity of fermentative yeasts yielding a final product of lower acidity and slightly higher pH. Among the LAB found in black olive fermentations, species of *Lc. mesenteroides*, *Lb. brevis*, *Lb. plantarum* and *Lb. pentosus* have been reported (Tassou, et al. 2002). Attempts to standardize the product quality shorten the fermentation time required for debittering, and ameliorating the risk of spoilage have been made by introducing bacterial starter cultures (Comi, et al. 2000; Servili, et al. 2006). Lactobacilli isolated from black table olive brines were evaluated for their salt tolerance, resistance to oleuropein and verbascoside, and ability to grow in modified filter-sterilized brines (Servili, et al. 2006). Among them, strain IMO was selected for its superior properties and further identified as *Lb. pentosus* by 16S rRNA gene sequencing followed by *recA* gene-based multiplex PCR. The use of strain of *Lb. pentosus* IMO as a starter yielded ready-to-eat, high-quality black table olives and reduced the debittering period considerably (Servili, et al. 2006).

5 Perspectives

Our current knowledge of the microbiological aspects of many traditional fermented vegetable foods still depends on data obtained by classical biochemical and physiological tests. By contrast, the application of molecular methods in the field of microbiology allows a better understanding of the ecology of food fermentations. Since the results are simple to interpret and mistakes in the identification of species due to the problems related to biochemical tests are avoided, dominant strains responsible for the main transformations during fermentations can be easily detected and identified. PCR-based fingerprinting methods such as RAPD-PCR, ARDRA, or ITS-PCR can be of great value to estimate the genetic diversity of

strains in food fermentations, and also to follow the proliferation capacity of selected strains intended to be used as starters. PCR-based fingerprinting methods have been successfully used to study the genetic diversity of LAB involved in fermentation of caper berries as well as “Almagro” eggplants, but little work has been done in other fields such as sauerkraut or table olive fermentations. Conceivably, further research in this direction should gather valuable knowledge on the microbial diversity involved in these processes, as well as existing differences related to geographic region and variations in homemade and industrial-processed olives.

The use of culture-independent methods is also a recent trend in the study of the microbial ecology of foods (Giraffa 2004). PCR-DGGE fingerprinting has been used to study microbial communities in different fermented foods including pozol, cassava, fermented Sausages, and dairy products (Ercolini 2004). However, the potential of this method to study the microbial communities of other traditional fermented foods such as “Almagro” eggplants, sauerkraut or table olives has not been exploited so far. Polyphasic approach studies, including culture-dependent and culture-independent methods such as denaturing gradient gel electrophoresis (DGGE), still need to be carried out to broaden our current knowledge of such fermentations. TTGE, which has been used to characterize other types of microbial populations (Bosshard, et al. 2000; Ogier, et al. 2002), is a technique that works on the same basic principle as DGGE, but without the requirement for a chemical denaturing gradient, thus producing more reproducible data (Yoshino, et al. 1991). Analysis of the profiles obtained by TTGE allowed the identification of the main groups of LAB involved in caper fermentation (Pérez Pulido, et al. 2005) as well as such African cereal-based fermented foods as *poto poto* and *dégué* (Abriouel, et al. 2006). In spite of the promising results obtained so far, further work is still needed to clarify the microbiological profiles of traditional fermented foods to achieve a broader picture of the microbial diversity and the genetic resources that these foods may hide. The risks of replacing traditional fermentation methods with industrial-scale fermentations, or a shift in the population’s consumption away from traditional fermented foods strengthens the need to pursue their study before these processes and resources are lost. This may be particularly relevant in the case of Mediterranean vegetable fermentations such as capers, “Almagro” eggplant, and homemade table olives.

References

- Abriouel, H., Ben Omar, N., Lucas López, R., Martínez-Cañamero, M., Keleke, S., Gálvez, A., 2006. Culture-independent analysis of the microbial composition of the African traditional fermented foods *poto poto* and *dégué* by using three different DNA extraction methods. *Int. J. Food Microbiol.* 111, 228–233.
- Alvarruiz, A., Rodrigo, M., Miquel, J., Girer, V., Fera, A., Vila, R., 1990. Influence of brining and packing conditions on product quality of capers. *J. Food Sci.* 55, 196–198.
- Ballesteros, C., Palop, M.L., Sánchez, I., 1999. Influence of sodium chloride concentration on the controlled lactic acid fermentation of ‘Almagro’ eggplant. *Int. J. Food Microbiol.* 53, 13–20.

- Ben Omar, N., Ampe, F., 2000. Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl. Environ. Microbiol.* 66, 3664–3673.
- Bosshard, P., Santini, Y., Grütter, D., Stettler, R., Bachofen, R., 2000. Bacterial diversity and community composition in the chemocline of the meromictic alpine Lake Cadagno as revealed by 16S rDNA analysis. *FEMS Microbiol. Ecol.* 31, 173–182.
- Buckenhüskes, H.J., 2001. Fermented Vegetables. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology: Fundamentals and Frontiers* (2nd Edition). ASM Press, Washington D.C, pp. 665–679.
- Comi, G., Manzano, M., Cocolin, L., Vailanti, P., Cantoni, C., Giomo, A., 2000. Valutazione dell'attività di starters microbici nella preparazione delle olive da mensa. *Ind. Aliment. (Pinerolo, Italy)*. 39, 1258–1265.
- Coton, E., Coton, M., Levert, D., Casaregola, S., Sohier, D., 2006. Yeast ecology in French cider and black olive natural fermentations. *Int. J. Food Microbiol.* 108, 130–135.
- Daeschel, M.A., Anderson, R.E., Fleming, H.P., 1987. Microbial ecology of fermenting plant materials. *FEMS Microbiol. Rev.* 46, 357–367.
- Durán, M.C., García, P., Brenes, M., Garrido, A., 1993. *Lactobacillus plantarum* survival during the first days of ripe olive brining. *Syst. Appl. Microbiol.* 16, 153–158.
- Durán Quintana, M.C., García García, P., Garrido Fernández, A., 1999. Establishment of conditions for green table olive fermentation at low temperature. *Int. J. Food Microbiol.* 51, 133–143.
- Ercolini, D., Moschetti, G., Blaiotta, G., Coppola, S., 2001. The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water buffalo mozzarella cheese production: bias of culture-dependent and culture-independent analyses. *Syst. Appl. Microbiol.* 24, 610–617.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Meth.* 56, 297–314.
- Ercolini, D., Villani, F., Aponte, M., Mauriello, G., 2006. Fluorescence *in situ* hybridization detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations. *Int. J. Food Microbiol.* 112, 291–296.
- Fernández Díez, M.J., 1983. Olives. In: Rehm, H.-J., Reed, G. (Eds.), *Biotechnology: Food and Feed Production with Microorganisms*. Verlag, Florida, pp. 379–397.
- Fernández González, J., Garrido Fernández, A., García García, P., Brenes Balbuena, M., Durán Quintana, M.C., 1992. Características del proceso fermentativo durante la conservación de aceitunas de la variedad Hojiblanca, destinadas a la elaboración del tipo negras. *Grasas Aceites* 43, 212–218.
- Fernández González, M.J., García, P., Garrido Fernández, A., Durán Quintana, M.C., 1993. Microflora of the aerobic preservation of directly brined green olives from Hojiblanca cultivar. *J. Appl. Bacteriol.* 75, 226–233.
- Fleming, H.P., 1982. Fermented vegetables. In: Rose, A.H. (Ed.), *Economic Microbiology Fermented Foods*. Academic Press, New York.
- Fleming, H.P., McFeeters, R.F., Humphries, E.G., 1988. A fermentor for study of sauerkraut fermentation. *Biotechnol. Bioeng.* 31, 189–197.
- Fleming, H.P., Kyung, K.H., Breidt, F., 1995. Vegetable fermentations. In: Rehm, H.J., Reed, G. (Eds.), *Biotechnology*, vol. 9. VCH Publishers, New York, pp. 629–661.
- Fox, P.F., 1993. Cheese: an overview. In: Fox, P.F. (Ed.), *Cheese; Chemistry, Physics and Microbiology*. Chapman & Hall, London, pp. 1–36.
- Garrido Fernández, A., García García, P., Brenes Balbuena, M., 1995. Olive fermentations. In: Rehm, H.J., Reed, G. (Eds.), *Enzymes, biomass, food and feed*. VCH, New York, pp. 593–627.
- Garrido Fernández, A., Fernández Díaz, M.J., Adams, M.R., 1997. *Table Olives: Production and Processing*. Chapman & Hall, London.
- Giraffa, G., 2004. Studying the dynamics of microbial populations during food fermentation. *FEMS Microbiol. Rev.* 28, 251–260.

- Harris, L.J., 1998. The microbiology of vegetable fermentations. In: Wood, B.J.B. (Ed.), *Microbiology of Fermented Foods*, vol. 1. Blackie Academic & Professional, London, pp. 45–72.
- Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts, F.M., 1993. Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawè, a fermented maize dough from Benin. *Int. J. Food Microbiol.* 18, 279–287.
- Jay, J.M., Loessner, M.J., Golden, D.A., 2005. *Modern Food Microbiology* (7th Edition), Springer.
- Jiménez-Díaz, R., Rios-Sanchez, R.M., Desmaseaud, M., Ruiz-Barba, J.L., Piard, J.C., 1993. Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Appl. Environ. Microbiol.* 59, 1416–14.
- Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 49, 209–224.
- Kotzekidou, P., 1997. Identification of yeasts from black olives in rapid system microtitre plates. *Food Microbiol.* 14, 609–616.
- Kunene, N.F., Geomaras, I., von Holy, A., Hastings, J.W., 2000. Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. *Appl. Environ. Microbiol.* 66, 1084–1092.
- Leal-Sánchez, M.V., Ruiz-Barba, J.L., Sánchez, A.H., Rejano, L., Jiménez-Díaz, R., Garrido, A., 2003. Fermentation profile and optimization of green olive fermentation using *Lactobacillus plantarum* LPCO10 as a starter culture. *Food Microbiol.* 20, 421–430.
- Leone, F.G., 2000. The globalization of the olive oil market and the competitive position of the sector in Italy: an international comparison. *Olivae* 83, 10–14.
- Luna, F., Pérez, M., 1985. La Tapanera o alcaparra. Cultivo y aprovechamiento. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Spain.
- Maldonado, A., Ruiz-Barba, J.L., Jiménez-Díaz, R., 2003. Purification and genetic characterization of plantaricin NC8, a novel co-culture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. *Appl. Environ. Microbiol.* 69, 383–389.
- Maldonado A., Jiménez-Díaz R., Ruiz-Barba, J.L., 2004. Induction of plantaricin production in *Lactobacillus plantarum* NC8 after co-culture with specific gram-positive bacteria is mediated by an autoinduction mechanism. *J. Bacteriol.* 186, 1556–1564.
- Marquina, D., Peres, C., Caldas, F.V., Marques, J.F., Peinado, J.M., Spencer-Martins, I., 1992. Characterization of the yeast population in olive brines. *Lett. Appl. Microbiol.* 14, 279–283.
- Marquina, D., Toufani, S., Llorente, P., Santos, A., Peinado, J.M., 1997. Killer activity in yeast isolates from olive brines. *Adv. Food Sci.* 19, 41–46.
- Meroth, C. B., Walter, J., Hertel, C., Brandt, M.J., Hammes, W.P., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69, 475–482.
- Miambi, E., Guyot, J.P., Ampe, F., 2003. Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 82, 111–120.
- Montaño, A., de Castro, A., Rejano, L., 1992. Transformaciones bioquímicas durante la fermentación de productos vegetales. *Grasas Aceites* 43, 352–360.
- Mugula, J.K., Ninko, S.A.M., Narvhus, J.A., Sorhaug, T., 2003. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. *Int. J. Food Microbiol.* 80, 187–199.
- Ogier, J.-C., Son, O., Gruss, A., Tailliez, P., Delacroix-Buchet, A., 2002. Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 68, 3691–3701.
- Özcan, M., 1999. Pickling and storage of caperberries (*Capparis* spp.). *Z Lebensm Unters Forsch* A. 208, 379–382.
- Özcan, M., 2001. Pickling caper flower buds. *J. Food Quality* 24, 261–269.

- Özcan, M., Akgül, A., 1999a. Pickling process of capers (*Capparis* spp.) flower buds. *Grasas y Aceites* 50, 94–99.
- Özcan, M., Akgül, A., 1999b. Storage quality in different brines of pickled capers (*Capparis* spp.) flower buds. *Grasas y Aceites* 50, 269–274.
- Oyewole, O.B., Odunfa, S.A., 1990. Characterization and distribution of lactic acid bacteria in cassava fermentation during fufu production. *J. Appl. Bacteriol.* 68, 145–152.
- Palop, M.L.I., Ballesteros, C., 2000. Fabricación de berenjenas de Almagro a partir de frutos congelados. Spanish Patent P-97 00957.
- Pederson, C.S., Albury, M.N., 1954. The influence of salt and temperature on the microflora of sauerkraut fermentation? *Food Technol.* 8, 1–5.
- Pederson, C.S., Albury, M.N., 1969. The sauerkraut fermentation. *N.Y. State Agric. Exper. Sta. Bull.* 824.
- Pérez Pulido, R., Ben Omar, N., Abriouel, H., Lucas López, R., Martínez Cañamero, M., Gálvez, A., 2005. Microbiological study of lactic acid fermentation of caper berries by molecular and culture-dependent methods. *Appl. Environ. Microbiol.* 71, 7872–7879.
- Pérez Pulido, R., Ben Omar, N., Abriouel, H., Lucas López, R., Martínez Cañamero, M., Guyot, J.P., Gálvez, A., 2007. Characterization of lactobacilli isolated from caper berry fermentations. *J. Appl. Microbiol.* 102, 583–590.
- Pérez Pulido, R., Abriouel, H., Ben Omar, N., Lucas López, R., Martínez Cañamero, M., Gálvez, A., 2006. Plasmid profile patterns and properties of pediococci isolated from caper fermentations. *J. Food Protect.* 69, 1178–1182.
- Plengvidhya, V., Breidt Jr., F., Fleming, H.P., 2004. Use of RAPD-PCR as a method to follow the progress of starter cultures in sauerkraut fermentation. *Int. J. Food Microbiol.* 93, 287–296.
- Randazzo, C.L., Restuccia, C.A., Romano, D., Caggia, C., 2004. *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. *Int. J. Food Microbiol.* 90, 9 – 14.
- Robinson, R.K., 1988. Development in Food Microbiology, vol. 3. London.
- Ruiz-Barba, J.L., Cathcart, D.P., Warner, P.J., Jiménez-Díaz, R., 1994. Use of *Lactobacillus plantarum* LPCO10, a bacteriocin producer, as a starter culture in Spanish style green olive fermentations. *Appl. Environ. Microbiol.* 60, 2059–2064.
- Sánchez, I., Palop, M.L.I., Ballesteros, C., 2000. Biochemical characterization of lactic acid bacteria isolated from spontaneous fermentation of “Almagro” eggplant. *Int. J. Food Microbiol.* 59, 9 – 17.
- Sánchez, I., Palop, L.I., Ballesteros, C., 2003. Identification of lactic acid bacteria from spontaneous fermentation of “Almagro” eggplants by SDS-PAGE whole cell protein fingerprinting. *Int. J. Food Microbiol.* 82, 181–189.
- Sánchez, I., Seseña, S., Palop, L.I., 2004. Polyphasic study of the genetic diversity of lactobacilli associated with “Almagro” eggplants spontaneous fermentation, based on combined numerical analysis of Randomly Amplified Polymorphic DNA and Pulsed- Field Gel Electrophoresis patterns. *J. Appl. Microbiol.* 97, 446–458.
- Sánchez, I., Seseña, S., Poveda, J.M., Cabezas, L., Palop, L.I., 2005. Phenotypic and genotypic characterization of lactobacilli isolated from Spanish goat cheeses. *Int. J. Food Microbiol.* 102, 355–362.
- Servili, M., Settanni, L., Veneziani, G., Esposito, S., Massitti, O., Taticchi, A., Urbani, S., Montedoro, G.F., Coretti, A., 2006. The use of *Lactobacillus pentosus* IMO to shorten the debittering process time of black table olives (cv. *Itrana* and *Leccino*): A pilot-scale application. *J. Agric. Food Chem.* 54, 3869–3875.
- Seseña, S. 2005. Caracterización tecnológica de cepas autóctonas y selección de cultivos iniciadores para la fermentación de la berenjena de Almagro. PhD Thesis. Universidad de Castilla-La Mancha, Toledo, Spain.
- Seseña, S., Sánchez, I., González Viñas, M.A., Palop, L.I., 2001. Contribution of starter culture to the sensory characteristics of fermented “Almagro” eggplants. *Int. J. Food Microbiol.* 67, 197–205.

- Seseña, S., Sánchez, I., González Viñas, M.A., Palop, L.I., 2002. Effect of freezing on the spontaneous fermentation and sensory attributes of "Almagro eggplants". *Int. J. Food Microbiol.* 77, 155–159.
- Seseña, S., Sánchez, I., Palop, L.I., 2004. Genetic diversity (RAPD-PCR) of lactobacilli isolated from "Almagro" eggplant fermentations from two seasons. *FEMS Microbiol. Lett.* 238, 159–165.
- Seseña, S., Sánchez, I., Palop, L.I., 2005. Characterization of *Lactobacillus* strains and monitoring by RAPD-PCR in controlled fermentations of "Almagro" eggplants. *Int. J. Food Microbiol.* 104, 325–335.
- Sugihara, T.F., 1985. Microbiology of Bread Making, In: Wood, B.J.B. (Ed.), *Microbiology of Fermented Foods*. Elsevier, Applied Science Publishers, UK.
- Tamang, J.P., Sarkar, P.K., 1996. Microbiology of mesu, a traditional fermented bamboo shoot product. *Int. J. Food Microbiol.* 29, 49–58.
- Tassou, C.C., Panagou, E.Z., Katsaboxakis, K.Z., 2002. Microbiological and physicochemical changes of naturally black olives fermented at different temperatures and NaCl levels in the brines. *Food Microbiol.* 19, 605–615.
- Yoshino, K., Nishigaki, K., Husimi, Y., 1991. Temperature sweep gel electrophoresis: a simple method to detect point mutations. *Nucl. Acids Res.* 19, 3153.

Chapter 6

Wine Fermentation

David A. Mills, Trevor Phister, Ezekial Neeley, and Eric Johannsen

Abstract The molecular biology revolution has brought forth significant new advances with application in microbiological analysis during wine production and storage. For example, traditional methods for microbial strain identification have been mostly supplanted in favor of ribosomal RNA-based methods for speciation of cultured yeast and bacterial populations in wine. Moreover culture-independent molecular methods now allow for more rapid profiling of complex populations, or quantification of targeted species, thereby enhancing the information available to the winemaker. Finally, the availability of microbial genome sequences provides a wealth of new opportunities to understand and exploit the microorganisms in wine, as well as identify the key genetic factors underlying wine flavor development or depreciation. In general, advances in molecular biology are fundamentally changing how scientists and winemakers assess the microbial ecology of winemaking, providing new insight into the wonderfully complex conversion of grape juice to wine.

1 Introduction

The conversion of grape juice to wine is a biotechnological tradition dating back to the dawn of civilization. Throughout the ages numerous winemaking strategies were developed resulting in the range of wine products, from champagne to port, available today. However, since the time of Pasteur (1873) the microbial contribution to the production of wine has become a subject of research and, often, debate. Wine composition and quality are functions of many different intrinsic and extrinsic variables, many of which are microbiologically mediated. A large diversity of microbes are inherent to winemaking including various yeasts, bacteria and fungi. Prominent in this process are *Saccharomyces* species (predominantly *S. cerevisiae*), which dominate the alcoholic fermentation, and the lactic acid bacteria (LAB), which carry out the malolactic conversion. Efforts to determine the population size and potential impact of different microbes on the winemaking process are critical to production of a flavorful product. Spoilage is considered growth of organisms that are unwanted at any particular place and time in the winemaking process (Sponholz 1993). Thus the same microorganism can be both beneficial and detrimental to the

winemaking process. For example, growth of *S. cerevisiae* is required during the alcoholic fermentation, but growth can be detrimental if it occurs in a finished, and bottled, wine.

Interestingly, both academics and winemakers have good reason to be interested in the microbial ecology of the winemaking process. From an academic perspective wine represents an ideal landscape in which to study basic concepts of microbial ecology. Several factors promote this view. First and foremost, wine is a liquid medium that allows samples to be properly mixed prior to analysis, thus ensuring a representative sampling. This contrasts with the situation for those studying microbial growth on solid surfaces (e.g., barrel stave, grape surface or vineyard soil) in which the microbial populations are heterogeneous and spatially distributed across the surfaces. As a consequence, representative sampling of microbes on surfaces becomes a more statistically challenging process than sampling of a liquid medium like must or wine. A second reason why wine is an attractive platform for microbial ecology is the diversity of microbes present which enables one to witness a range of microbial interactions from commensalisms and neutralisms to antagonisms.

From the winemaker perspective, close monitoring of the microbial changes occurring throughout the winemaking process is beneficial for several reasons: to promote and guide yeast during the alcoholic fermentation, to verify the growth of the bacteria during the malolactic conversion, and ultimately to ensure the stability of the wine before bottling and storage (Delfini and Formica 2001). The evolution of undesired microbes during different stages of winemaking can produce volatile acidity, off-flavors and polysaccharide hazes, all of which can diminish the quality and acceptability of the final product (Sponholz 1993). Even prior to the onset of fermentation, the grapes themselves can be infected with molds, yeasts and bacteria that can enter and alter the fermentation in a negative fashion. Improper wine storage and handling post-fermentation can encourage microbiological faults, which can negatively impact wine quality. As a result the winemaker must conduct basic physical, chemical, sensory and microbiological analyses of musts and wines to assure wine quality.

Whether it is for an investigation of basic ecological concepts or for the applied goal of predicting possible wine spoilage, one must have accurate and reproducible methods for enumeration of various microbial constituents at different stages in wine production. Both indirect and direct approaches can be used to view these populations. In this review, we will summarize both approaches and comment on the future use of newer molecular tools to view the microbial diversity inherent in wine fermentations.

1.1 A Brief Overview of the Winemaking Process

Given the process of winemaking is ancient, it is not surprising that a multitude of winemaking styles and wine products abound, each of which can influence the microbial presence. In this section we will describe the core aspects of winemaking

and the important microbiological changes inherent to that process. More detailed descriptions of the many strategies employed in winemaking can be found elsewhere (Boulton, et al. 1996; Ribereau-Gayon, et al. 2000). A general schematic of common steps in red and white winemaking is presented in Fig. 6.1.

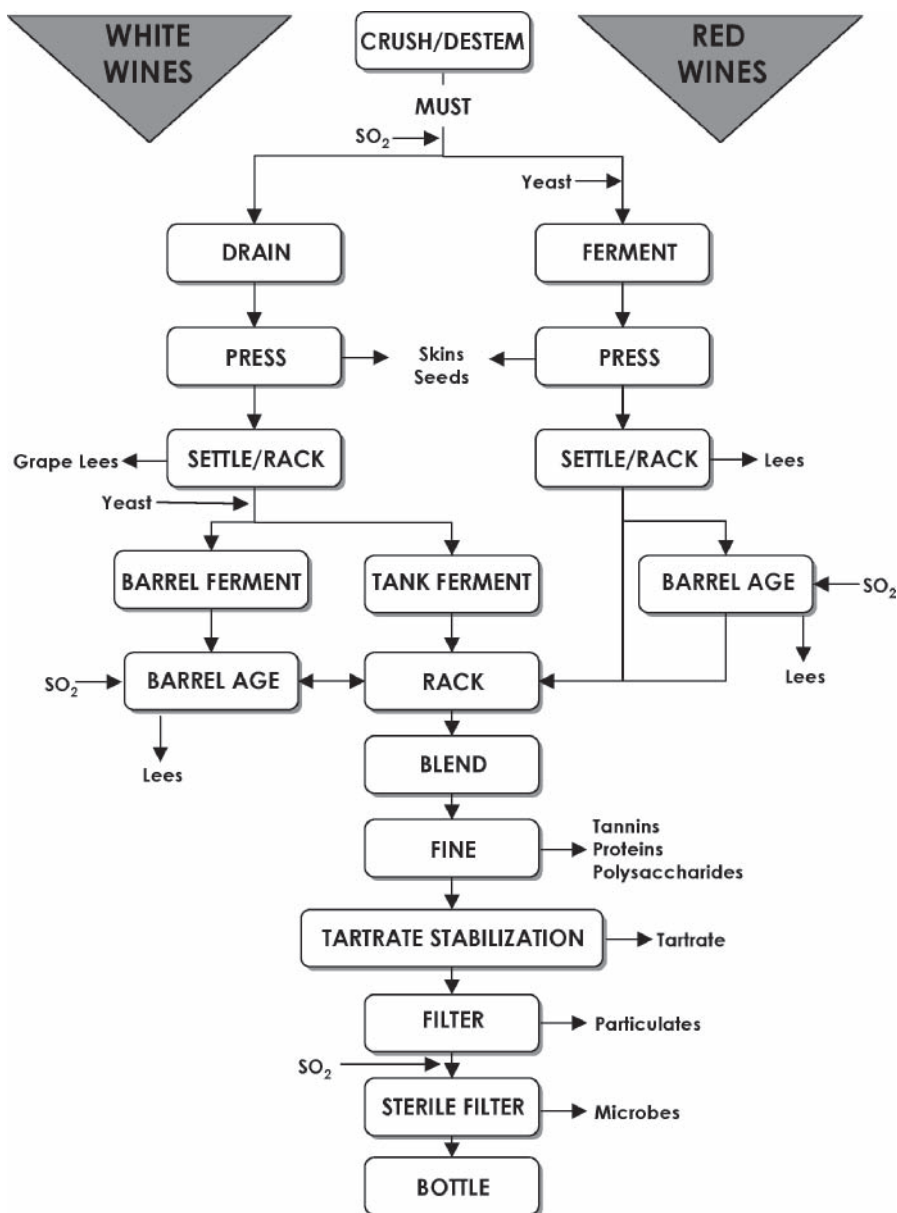


Fig. 6.1 General schematic for production of white and red wines

Winemaking begins with the collection and crushing of grapes. For white wines the grape juice is separated away from the skins and clarified via cold settling, filtration or centrifugation. The juice is then moved to a barrel or fermentation tank and the alcoholic fermentation is carried out by yeasts indigenous to the juice, or via inoculation of a selected *S. cerevisiae* starter culture. White wine fermentations are typically carried out for roughly one to two weeks at temperatures around 10 to 18°C. Upon consumption of available glucose and fructose, the main sugars in grape juice, the wine is considered “dry” and separated from the yeast and grape lees (sediment).

Red wines are produced slightly differently than white wines. After crushing the skins are left in the fermentation to allow for color extraction. Like white wines, the alcoholic fermentation commences either through the action of indigenous yeasts or via direct inoculation of a starter culture. During the fermentation the grape material tends to float to the top of the vat forming a “cap.” To better enable extraction of red pigments and to influence wine flavor, winemakers typically punch down the cap or pump juice from the bottom over the cap. After a suitable period of time, the wine is separated from the grape skins and the fermentation is completed in another vessel. As described for white wines, the red wine is now “dry” and devoid of the main juice sugars.

After the alcoholic fermentation, wines often are spontaneously, or purposely, taken through a malolactic fermentation in which the high level of malate in the juice is converted to lactate, mostly by indigenous or inoculated LAB. Unlike the alcoholic fermentation, the malolactic fermentation is a stylistic consideration by the winemaker, who, through use of antimicrobial additions (primarily sulfur dioxide) or filtration may choose to prevent this fermentation from initiating.

Once the wine has been taken through the alcoholic and, if desired, the malolactic fermentation, the wine is often stored in tanks or barrels to allow flavor development. The residence time for storage is primarily determined by the style of wine and winemaker choice. Often white wines are not stored for long periods of time while reds are frequently stored in oak barrels for several years. While the average wine contains approximately 13 percent ethanol, the alcohol by itself does not preclude future spoilage. Consequently winemakers must take great care to prevent exposure of the wine to oxygen, which can encourage microbial growth, as well as judiciously use antimicrobials (again, primarily sulfur dioxide) to prevent microbial spoilage.

1.2 Microorganisms in the Winery Environment

The initial environment that affects the microbial makeup of a wine fermentation is that of the vineyard. Although a drastically different environment than juice or wine, the types of microbes present on grapes will have an impact on the ensuing ecology in the wine fermentation, particularly in the early stages. Microorganisms appear to colonize around the grape stomata where small amounts of exudate are secreted

(Ribereau-Gayon, et al. 2000). The apiculate yeasts, *Hanseniaspora* and *Kloeckera*, its asexual anamorph, are the most prevalent vineyard yeasts and typically represent over half the yeast flora on grapes (Pretorius, et al. 1999). Other yeast genera present on berries include: *Metschnikowia*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces* and *Torulopsis* (Barnett, et al. 1972; Rosini, et al. 1982; Moore, et al. 1988). Also present in the vineyard are numerous other yeasts, some of which have an impact on wine: *Sporobolomyces*, *Kluyveromyces*, and *Hansenula* (Davenport 1974). *Saccharomyces* species are relatively scarce among healthy berries (Vaughan-Martini and Martini 1995; Mortimer and Polsinelli 1999). On damaged berries, *Saccharomyces* is present at significant but low levels (10^5 to 10^6 CFU per berry), compared to total microbial population levels of 10^7 to 10^8 CFU per berry (Mortimer and Polsinelli 1999). Mortimer (1999) suggested honey bees, wasps, and fruit flies as likely vectors for carrying and spreading *Saccharomyces* and other yeasts among damaged grapes.

Filamentous fungi also colonize the grape surface. Mold and mildew damage can influence the grape and wine microbial ecology in several ways. Of much interest is the mold *Botrytis cinerea*, known as noble rot or gray mold rot, depending on the degree of infection. Noble rot appears to occur on healthy berries where fungal hyphae penetrate cracks surrounding the stomatal opening or fissures in the cuticle; gray mold rot, on the other hand, infests damaged berries (Ribereau-Gayon, et al. 1980). The former mode of infection is associated with the high quality of sweet wines of limited production, such as Auslese/Beerenauslese/Trockenbeerenauslese, Tokay, Sauternes and others from California, South Africa and Japan (Dittrich 1991). Dehydration caused by increased porosity of the grape skin results in higher sugar concentration (as much as twice as high) in the resulting must. *Botrytis* sp. infection metabolizes sugars and malic and tartaric acids, reducing the total sugar content somewhat and raising the pH of the wine (Hofmann 1968). Moreover, *Botrytis* sp. infection encourages the proliferation of acetic acid bacteria (AAB) (Joyeux, et al. 1984) and yeasts (Le-Roux, et al. 1973) on grapes which, in turn, can affect the chemical composition and microbial makeup of the must/juice. Such damaged berries, whether resulting from mold attack, precipitation-induced swelling, hail or other pests are considered "very rich depositories" of microorganisms (Mortimer and Polsinelli 1999).

A distinct turning point occurs between the vineyard and the winery. As soon as the grapes are handled they become exposed to a new pool of organisms. The transfer of molds, yeasts and bacteria from equipment and surfaces represents the potential introduction of "resident" winery microbes to the grapes (Peynaud and Dimerco 1959) and, conversely, new sources of substrate are made available to existing microbes on the grape. The microbial populations present on equipment surfaces will vary according to the extent of sanitation employed on everything from picking knives, mechanical harvesters and grape bins, to crushers, tanks, hoses and pumps, to the walls and floors. Various species from the genera *Saccharomyces*, *Candida*, *Pichia*, and *Brettanomyces* were associated with winery equipment and surfaces (Peynaud and Dimerco 1959). Variables—with respect to time, temperature, and handling—of grape transport between the vineyard and the

winery likely affect the ecology of the grapes. Since some damage to the grapes occurs, especially with machine-harvested fruit, activity of microorganisms in the resulting juice probably occurs.

As a result of grape microflora and grape processing, the resultant juice, fermenting must and wine are complex microbial ecologies hosting a diverse collection of yeast and bacteria. The dominant yeast responsible for alcoholic fermentation—the bioconversion of six-carbon sugars to ethanol—is *S. cerevisiae*. However, juice and wine may also contain a wide array of yeast genera including *Metschnikowia*, *Dekkera* (anamorph *Brettanomyces*), *Pichia*, *Candida*, *Hanseniaspora* (anamorph *Kloeckera*), *Kluyveromyces*, *Issatchenkia*, *Torulaspora*, *Debaryomyces*, *Saccharomycodes*, *Zygosaccharomyces* and *Schizosaccharomyces* among others (Fleet 2003; Loureiro and Malfeito-Ferreira 2003). Depending on the acidity and the nutrient, oxygen and ethanol concentrations in the juice or wine, the active bacteria present typically include both LAB and AAB (Osborne and Edwards 2005). Other bacteria such as bacilli, clostridia, actinomyces or streptomyces have been identified in the wine environment, however, these represent relatively rare occurrences.

Once the grapes have been thoroughly crushed (or pressed), a new stage begins in the selection of yeast and bacterial species. The change in environment results in a change in selective pressures. Furthermore, enological practices alter the conditions of the juice. Most notably, sulfur dioxide is widely utilized in winemaking to control populations of microorganisms. Most wine-related bacteria and many yeasts are sensitive to molecular SO_2 . Molecular SO_2 is present in solution in pH-dependent equilibrium with bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) ions. The proportion of the molecular SO_2 species is higher at lower pH; for instance, at pH 3.0, the proportion of molecular SO_2 in solution is 10 times higher than at pH 4.0 (Boulton, et al. 1996). *Saccharomyces* species are relatively resistant to SO_2 , as are the apiculate yeasts (Stratford, et al. 1987). The bacteriolytic enzyme lysozyme is another antimicrobial additive increasingly used to reduce bacterial populations (Fuglsang, et al. 1995), however, its use on a large production scale is limited by cost (Pretorius 2000).

1.3 Yeast Ecology during Fermentation

As fermentations proceed, metabolites and other products generated by yeasts can impact the performance of other organisms. The products of fermentation, CO_2 and ethanol, are prime examples. The evolution of CO_2 can inhibit yeast growth at 15 g/L (Dittrich 1991), but it has the primary effect of excluding O_2 from the fermenting medium. This action, especially in combination with alcohol, prevents the growth of aerobic organisms generally associated with spoilage. Different yeast species and strains have varying sensitivities to ethanol. *S. cerevisiae* species, as would be expected, have high ethanol tolerance—it can generally ferment to 15 percent to 16 percent ethanol (Gao and Fleet 1988; Dittrich 1991). Gao and Fleet (1988) showed

that some strains of *C. stellata* had relatively high ethanol tolerance, as well, while *Kloeckera* sp. exhibited fairly low tolerance. Higher temperatures appear to decrease ethanol tolerance. Some non-*Saccharomyces* yeasts found in wine are capable of producing ethanol – namely *Torulaspora delbrueckii*, *Saccharomycodes ludwigii* at moderate to high levels, *C. stellata* at low to moderate levels, and the apiculate yeasts at low levels – with production limits a likely indication of ethanol tolerance (Ciani and Maccarelli 1998). The resistance of *S. cerevisiae* to its metabolic products, especially ethanol, work to select it as a dominant organism in fermentations.

In addition to ethanol, yeasts produce other metabolites that broadly inhibit cell growth. Medium chain fatty acids, octanoic and decanoic acids, act by interfering with plasma membrane integrity (Alexandre and Charpentier 1998; Bisson 1999). Wine yeast also produce killer factors which can impact the survival of other yeasts within the same environment (van Vuuren and Jacobs 1992). Finally different strains of *S. cerevisiae* have been shown to produce anywhere from 25 to 100 ppm of SO₂ (Thornton 1991).

Obviously a major factor affecting microbial composition in wine fermentations is the practice of inoculation with commercial or otherwise selected strains of *S. cerevisiae*. Inoculation can be particularly effective in combination with SO₂ in reducing non-*Saccharomyces* populations and promoting the growth of *S. cerevisiae* (Constanti, et al. 1998; Egli, et al. 1998).

1.4 The Lactic Acid Bacteria

The LAB involved in wine are comprised of acid and ethanol-tolerant strains primarily from four genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus* (formerly *Lc. oeni*) (Sponholz 1993; Lonvaud-Funel 1999; Osborne and Edwards 2005). These microbes are commonly found on grapes and in the winery environment. Newly fermented wines contain low populations of LAB, usually less than 10³ CFU per mL (Davis, et al. 1985), however, damage to the grapes increases this number by several orders of magnitude. Three main factors that dictate the extent of LAB growth in wine are pH, ethanol and antimicrobial additions such as SO₂ or lysozyme. These latter additions purposely reduce LAB concentrations to enable proper growth of *S. cerevisiae* and/or to microbially stabilize the wine. Wine pH also strongly influences which LAB species will be present. Higher pH wines (above pH 3.5) often harbor species of *Lactobacillus* and *Pediococcus*, both during and after fermentation, while lower pH wines (< 3.5) typically only contain *O. oeni* (Fleet 1998; Osborne and Edwards 2005).

Ethanol production from the dominant *S. cerevisiae* population also serves to reduce all LAB populations in the first few weeks of the alcoholic fermentation. However, as the wine is stored, the ability of ethanol-tolerant LAB to emerge increases. Growth substrates can be available at this stage as a consequence of yeast cell lysis and release of nutrients into the wine (Lonvaud-Funel 1999).

1.5 The Acetic Acid Bacteria

AAB are Gram-negative obligate aerobes that produce acetic acid, acetaldehyde and ethyl acetate from both glucose and ethanol (Ribereau-Gayon, et al. 2000). AAB are spoilage organisms commonly found in wine, beer and cider (Kersters, et al. 2006). Those found in wine belong to the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Osborne and Edwards 2005). Previously lumped together under the genus *Acetobacter*, *Gluconobacter* was given its own genus because of its poor growth in beer and its ability to produce large amounts of gluconic acid from glucose (Adams 1998).

Damaged grapes often contain significant populations of *Gluconobacter* (Fleet 1998; Du Toit and Lambrechts 2002). In addition, production of gluconic acid and other carbonyl-containing compounds by *Gluconobacter* sp. can bind to SO₂, thus lowering the overall efficacy of sulfite additions (Barbe, et al. 2001). Therefore, from a winery's perspective, *Gluconobacter* sp. growth poses the biggest threat to juices and *Acetobacter* sp. to finished wines (Du Toit and Lambrechts 2002). Because they are aerobic bacteria, minimizing air contact of static wine is an effective means of controlling AAB. However, static populations of AAB will start multiplying again with exposure to air—as found in the pumping and transferring of wine (Sponholz 1993). Bartowsky and co-workers found *A. pasteurianus* to be a major culprit in the production of acetic acid in bottles of red wine that had been stored upright (Bartowsky, et al. 2003).

2 Culture-independent Studies on Wine Microbial Ecology

Most approaches to identify and enumerate microbes in wine involve enrichment techniques (Boulton, et al. 1996; Fugelsang 1997). Such methods are considered “indirect” because one is not enumerating the original cells in the sample, but their progeny, as enriched in a specific medium. Various texts (Boulton, et al. 1996; Fugelsang 1997) describe both general and selective growth media for plating yeasts and bacteria from wine. Unfortunately, plating and enrichment procedures are time consuming as colonies for some wine-related microbes take up to a week or more to appear on a plate. Additionally, once colonies do appear on a plate, definitive identification of the microbe requires further testing. More importantly, culture-based techniques typically underestimate the size and diversity of a population as sublethally injured or viable, but non-culturable (VBNC) cells, common in wine, may fail to grow on plates (Kell, et al. 1998; Millet and Lonvaud-Funel 2000). Understanding this difference between a true total cell count and a culturable population is important as VBNC or injured cells are still metabolically active.

At present a true VBNC state is only associated with a bacterial response to adverse environmental conditions such as starvation, changes in pH or the presence of antimicrobials (Barer and Harwood 1999). Alternatively, the non-culturable cells

may be sublethally injured by the chemicals used in wine such as sorbate, sulfite or ethanol, thus losing the ability to grow on standard culture media (Stevenson and Graumlich 1978; Fleet 1990; Davidson 1997; Fleet and Mian 1998). In either case, VBNC or sublethally injured organisms may still play a role in wine spoilage. Thus the examination of these states in wine-related microbes may help explain some of the known spoilage problems where correlations between spoilage and plating of specific microorganisms are difficult to derive. One such example is the production of the phenolic taints by *Brettanomyces* sp. (teleomorph *Dekkera*). *Brettanomyces* sp. have been shown to produce 4-ethylphenol from phenolic acids, at higher levels than other microorganisms present in the wine fermentations (Chatonnet, et al. 1995). However, it has been difficult to correlate the amount of 4-ethylphenol to the population size of *Brettanomyces* sp. in the wine. In some cases, when *Brettanomyces* sp. are not detected at significant levels, the compounds are still detected at high levels (Chatonnet, et al. 1995; Rodrigues, et al. 2001; Fugelsang and Zoecklein 2003). Another example is provided by Coton, et al. (1998) in studies of *O. oeni*. They inoculated *O. oeni* strain 9204 into a red wine after MLF and an addition of sulfite. After one month no colonies were detected, however, *O. oeni*-derived histidine decarboxylase activity was still detectable.

While the VBNC state or sublethal injury may provide an explanation for cases such as wine spoilage by *Brettanomyces*, few studies have examined the possible VBNC state in wine-related microbes. While the ability of bacteria to enter the VBNC state has been documented in many different environments, a number of studies also suggest that a VBNC state may exist in yeasts. First, Rodrigues and Kroll (1985), using the direct epifluorescent filter technique (DEFT) on *S. cerevisiae*, found that cell counts using acridine orange dye correlated well with plating results of non-stressed cells, but heat-treated samples gave higher counts compared to plating results. This observation, while not explained as a VBNC state, is similar to the higher direct viable counts of stressed cells seen by Xu, et al. (1982) that lead to the VBNC theory.

Regardless of whether non-culturable cells in wine are truly VBNC or simply sublethally injured, the fact that these cells continue to influence wine flavor and palatability argues the need for use of culture independent or "direct" analysis methods to assess the true population.

Various direct approaches have been developed for enumerating microbes in wine. The technique employed most often is simple microscopy from which wine-makers can readily differentiate yeast from bacteria, as well as determine microbial concentrations directly in wine with the use of counting chambers (Amerine and Kunkee 1968). Microscopic analysis, combined with use of simple stains such as methylene blue (Borzani and Vairo 1958), enables colorimetric differentiation of live versus dead yeast cells.

Another direct technique used by the wine industry to score microbial populations employs the enzyme luciferase to assay for microbial-borne ATP (de Boer and Beumer 1999; Gracias and McKillip 2004). This general approach has been adapted to various commercial systems for use in wineries. While this approach is useful in specific applications, such as to assess winery surfaces after cleaning, the

method also can detect ATP from other, non-microbial, biological sources and, thus, is problematic for use directly in wine fermentations.

Fluorescent dyes have also been employed to directly assess yeast viability in wine. Use of flow cytometry enables information on cell size and shape to be obtained by light scattering. In addition cell viability can be assessed directly using fluorescent dyes to view the metabolic state of yeast and bacteria in wine (Malacrino, et al. 2001; Boyd, et al. 2003; Chaney, et al. 2006; Herrero, et al. 2006). Flow cytometry can also be useful if, instead of stains, DNA probes or antibodies specific for a particular microbial species are used (Graca da Silveira, et al. 2002). This would allow not only the enumeration of targeted microbes but, coupled with a live/dead marker, also the percentage of living cells versus dead.

One particularly successful direct analysis application is the use of direct epifluorescence technique (DEFT) in which microbial-based cleavage of a fluorescent substrate enables direct counting of viable cells through a fluorescent microscope. Using this method Millet and Lonvaud-Funel (2000) first demonstrated significant non-culturable populations of both bacteria and yeast in aging wine, detecting at least 100-fold higher viable cell numbers using DEFT by comparison to that obtained by plating. They also demonstrated that both *A. aceti* and *P. damnosus* inoculated into wine maintained a higher viable cell population determined by DEFT, compared to plating. Interestingly, most of the non-culturable *A. aceti* population recovered the ability to grow on plates after aeration (Millet and Lonvaud-Funel 2000). In a later study, du Toit, et al (2005) used DEFT to demonstrate that *A. aceti* could survive for up to 71 days under anaerobic conditions in sulfited wine. These AAB exhibited a 100-fold difference between plating and DEFT counts and, upon addition of oxygen, the *A. aceti* populations became culturable. The yeasts *C. stellata*, *S. cerevisiae*, *Z. bailii* and *Rhodotorula mucilaginosa* were also found to enter the non-culturable state after addition of SO₂ in botrytized wine (Divol and Lonvaud-Funel 2005). Only *Z. bailii* and *R. mucilaginosa* were resuscitated in laboratory conditions. The fermentative yeasts *C. stellata* and *S. cerevisiae* were not able to recover, likely due to the presence of ethanol and a high osmotic environment (Divol and Lonvaud-Funel 2005). While these studies suggest that VBNC populations may exist in wine and that the VBNC state most likely plays a role in wine spoilage, more studies are needed to better understand the physiology of organisms in the VBNC state and their ability to effect wine during maturation.

2.1 Nucleic Acid-based Approaches

Numerous nucleic acid-based assays have been developed for directly characterizing microbes in wine. The first such techniques used probes generated to whole bacterial genomes to reveal specific LAB populations in wines (Sohier and Lonvaud-Funel 1998). More recently an array of probes used in fluorescence *in situ* hybridization (FISH) have been developed for direct analysis of LAB from wine (Blasco, et al. 2003). This includes specific probes for common wine species *O. oeni*, *P. damnosus*,

P. parvulus, *P. pentosaceus*, *Lb. plantarum*, *Lb. casei/paracasei*, *Lb. brevis*, *Lb. hilgardii* and most *Leuconostoc* species among others (Blasco, et al. 2003). The FISH approach was tested *in situ* on actual wine samples directly identifying *O. oeni* in 20 wines that had undergone the malolactic fermentation. While use of this method directly on wine has been rare, the general FISH approach is commonly used to characterize other environments. Moreover, the flexibility of the probe design to target whole taxa or select species enables useful representation of the possible populations present.

Other molecular survey techniques have been developed to profile total fungal or bacterial populations in natural environments (Head, et al. 1998). The more common of these methods employ amplification of ribosomal RNA genes by PCR followed by cloning (Head, et al. 1998), terminal restriction fragment length polymorphism (TRFLP) (Marsh 1999) or denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer and Smalla 1998).

2.2 Studies Employing PCR-DGGE

Of the popular survey methods, PCR-DGGE has been used the most to characterize both yeast and bacteria in the wine environment. PCR-DGGE was first applied to wine yeasts by Cocolin, et al (2000) who developed primers that amplified a portion of the D1-D2 loop of the yeast rRNA large subunit gene. That work demonstrated that the population shifts of different wine-related yeasts, such as *S. cerevisiae*, *M. pulcherrima*, *C. ethanolica*, and *K. apiculata*, in laboratory-based mixed-culture fermentations could be easily followed using PCR-DGGE. Importantly, this work revealed that PCR-DGGE could identify yeast populations that were at least 0.01 percent or higher of the dominant *Saccharomyces* sp. population, thereby defining the limits of detection for this method. This approach was then applied to follow yeast populations within a commercial sweet wine fermentation revealing the temporal presence of fungal species (*B. cinerea*) and several non-*Saccharomyces* yeasts, including *Metschnikowia* sp. and *Pi. anomala*, in the early stages of the fermentations along with the emergence and persistence of a dominant *S. cerevisiae* population (Cocolin, et al. 2001). This work also revealed the persistence of a *Candida* sp. DGGE signature throughout a complete wine fermentation (some 104 days later!). Additional studies on this commercial sweet wine fermentation revealed that PCR-DGGE signals for many non-*Saccharomyces* yeast populations could persist well into the fermentation and long after these yeasts could be identified on culture media (Mills, et al. 2002). This was particularly evident for the *Candida* sp. population (see Fig. 6.2), a species later determined to be *C. zemplinina* (Sipiczki 2003). DGGE signatures from both RNA and DNA templates directly purified from wine revealed *C. zemplinina* signatures persisted throughout the fermentation even when direct plating exhibited a relatively low number of cells. Direct RNA dot blot analyses using *C. zemplinina* specific probes revealed the size of that population at the end of the fermentation to be relatively

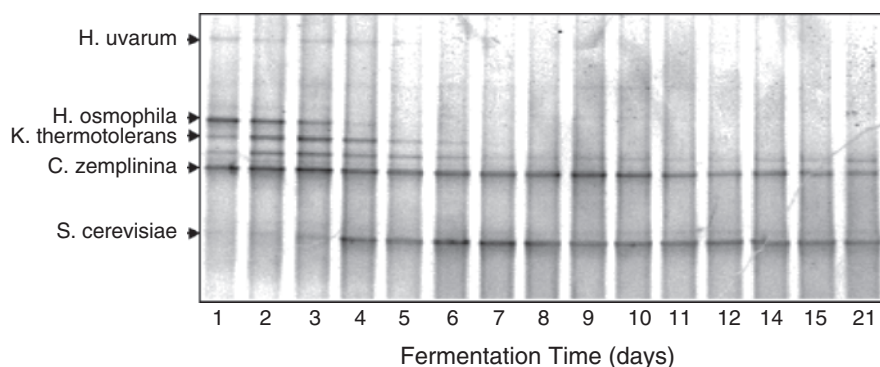


Fig. 6.2 Example of PCR-DGGE profiling of yeast constituents of a botrytized-wine fermentation. Individual bands were excised and sequenced to identify genera and species. Note that bands representing *C. zemplinina* and *S. cerevisiae* populations are evident throughout the fermentation

high ($>10^6$ cells per mL) when only 100–1000 CFU per mL could be detected by plating. These results provided some of the first evidence that metabolically active, yet non-culturable, yeasts persist in wine fermentations. Interestingly, when grown in isolation on grape juice, the *C. zemplinina* EJ1 isolate was shown to be exclusively fructophilic, a result which suggests a resource neutralism established between that strain and the dominant glucophilic *S. cerevisiae* culture present in these commercial fermentations.

Since the initial application of PCR-DGGE on wine several other groups have adopted this approach to directly profile yeasts in commercial fermentations. Cocolin, et al (2002) used PCR-DGGE to monitor a continuous wine fermentation, demonstrating a temporal occurrence of *M. pulcherrima* during the early stages of the fermentation and a stable presence of the inoculated *S. cerevisiae* culture throughout. Recently Renouf, et al (2006a) employed PCR-DGGE to follow wine production through alcoholic and malolactic fermentations at three wineries in France. In all three cases a relatively stable population of *Brettanomyces bruxelensis*, considered by many to be a spoilage yeast, was present after the alcoholic fermentation and appeared better able to prosper in the harsh environment of finished wine (e.g., low sugar and high ethanol).

While PCR-DGGE is a common approach to profile bacteria in other niches, the use of this approach in wine was complicated by inherent problems associated with primer specificity. Several groups have demonstrated how common primer sets used for bacterial PCR-DGGE mis-amplify eukaryotic DNAs (yeasts, molds or plants) (Lopez, et al. 2003; Dent, et al. 2004). To resolve this problem new 16S rRNA gene-based primers were developed (Lopez, et al. 2003), or alternative alleles such as the *rpoB* gene, were profiled (Dahllöf, et al. 2000). The latter provided an elegant approach since it obviated both the problems inherent in mis-amplification of non-bacterial DNAs, but also reduced the problems inherent to the heterogeneity among multiple copies of the 16S rDNA within the same bacteria. A limitation with this approach is the relative lack of *rpoB* gene sequences in public databases

restricts the ability to concretely identify DGGE bands upon re-sequencing. Renouf, et al. (2006b) recently employed *rpoB*-targeted primers to follow bacterial populations in wine from three wineries using PCR-DGGE. This work revealed significant differences in initial bacterial populations at each winery, likely a reflection of different winemaking practices and differential use of antimicrobial additions. In addition *O. oeni* was repeatedly and consistently observed throughout all the winery fermentations, even in fresh must samples. Finally, this approach revealed a secondary *Pediococcus* population at one winery after racking and sulfating of the wine, suggesting emergence of a spoilage population.

PCR-DGGE approaches have been applied less frequently to characterize the yeast or bacterial populations on wine grapes. Prakitchaiwattana, et al. (2004) found mostly the *Aureobasidium pullulans*, a ubiquitous environmental yeast, on undamaged grapes and *Metschnikowia* and *Hanseniaspora* sp., as well as *Au. pullulans*, on damaged grapes. The authors noted that PCR-DGGE was not as sensitive as plating and could not detect yeasts at population levels lower than 10⁴ CFU per gram (of grapes), however, they did note that a greater diversity of fungal species from grapes could be witnessed by DGGE. Recently Renouf and Lonvaud-Funel (2007) developed a modified enrichment method, followed by PCR-DGGE and other molecular methods, to demonstrate that the grapes may be a source for the spoilage yeast *Br. bruxellensis* (teleomorph *Dekkera bruxellensis*). This remarkable finding provides a clue as to a non-winery source for this problematic yeast.

2.3 Direct PCR Approaches

While the molecular profiling methods discussed above have provided a window from which to view all of the individual constituents of wine fermentation, both culturable and non-culturable, other PCR approaches directly assay for select population members. Endpoint PCR assays have been developed for several wine yeast and bacteria. Lopez, et al (2003) used a multiplex PCR approach amplifying different segments of the yeast *S. cerevisiae* COX1 gene to differentiate different starter strains. The authors then demonstrated they could employ the multiplex PCR directly on wine fermentation samples to assess implantation of a dominant starter culture. Ibeas, et al. (1996) developed a nested PCR method using primers designed to a putative RAD4 gene which readily detected *D. bruxellensis* and synonymous strains. The assay was employed directly on sherry wine samples to reveal the presence of *D. bruxellensis* in wine suspected to contain *Dekkera* sp. contamination. Cocolin, et al. (2004) used a similar approach designing specific primers to the *Br. bruxellensis* and *Br. anomalus* 26S rRNA gene to directly confirm *Brettanomyces* sp. contamination within wine using a single PCR reaction. The authors also showed that *Br. bruxellensis* and *Br. anomalus* were further resolved by a restriction digestion of the resultant amplicon. Cocolin, et al. (2003) also developed 26S rRNA gene PCR primers for specific amplification of *H. uvarum* and *C. zemplinina*. In that work the authors revealed a persistence of both RNA and DNA signatures for

H. uvarum and *C. zemplinina* in sulfited wine, even though no growth of either species was witnessed on plating media. While some researchers suggest that detection of RNA, even ribosomal RNA, is a signal of a metabolically active state—mostly due to the fact that RNA degrades more rapidly than DNA—the actual metabolic state of these cells remained unknown and the persistence of RNA signatures from dead cells in wine has yet to be examined. Thus the detection of *H. uvarum* and *C. zemplinina* RNA signatures in wine more than 20 days after no cognate colonies could be enriched on plates provides a useful example of how PCR approaches need to be viewed with caution since both live and dead cells may be detected.

Several groups have developed direct PCR methods to identify the specific bacteria in wine. Zapparoli, et al. (1998) and Bartowsky and Henschke (1999) independently developed direct PCR assays to identify the malolactic bacterium *O. oeni* using primers specific for the malate decarboxylase gene (*mleA*) or the 16S rRNA gene, respectively. In both cases the threshold for detection in wine was around 10^3 to 10^4 cells per mL. Others have used direct PCR to detect bacterial genes associated with a specific taint. Le Jeune, et al. (1995) developed PCR primers that amplify the gene encoding histidine decarboxylase (HDC), the cause of the biogenic amine histamine, from several LAB. Cotton, et al. (1998) then used this assay to survey 118 wines from Southwestern France and found nearly half of the wines surveyed possessed an amplifiable HDC allele. Gindreau, et al. (2001) used a similar strategy to detect exopolysaccharide-producing strains of *P. damnosus* with a detection limit in wine of 100 CFU per mL.

2.4 Real-time or Quantitative PCR (QPCR) Approaches

A more recent technique that has found wide application in wine fermentations is QPCR. In QPCR the logarithmic amplification of a DNA target sequence is linked to the fluorescence of a reporter molecule. Several different reporter formats exist for QPCR (Hanna, et al., 2005), however, a common reporter used for detection of wine-related organisms is the dye SYBR Green (Vitzthum and Bernhagen 2002). This fluorescent dye binds double stranded DNA molecules and has an excitation wavelength of about 250_{nm} and an emission wavelength around 497_{nm}. This fluorescence, which is read after each round of DNA amplification, may either be compared to an external standard curve known as absolute quantification or it may be compared to an internal or external control sample in a method known as relative quantification (Livak and Schmittgen 2001).

Relative quantification is primarily used to follow gene expression. While the use of relative quantification to analyze gene expression in wine-related microbes provides valuable insights into their biology, the use of absolute quantification is by far the most common type of QPCR employed in wine ecology. To date, QPCR detection methods have been developed for direct enumeration of wine-related microorganisms including *O. oeni* (Pinzani, et al. 2004), LAB (Neeley, et al. 2005), AAB (Gonzalez, et al. 2006), total yeasts (Hierro, et al. 2006), *D. bruxellensis* (Phister and Mills 2003;

Delaherche, et al. 2004), *S. cerevisiae* (Martorell, et al. 2005) and *Zygosaccharomyces* species (Casey and Dobson 2004; Rawsthorne and Phister 2006).

With QPCR, specific bacteria or non-*Saccharomyces* yeasts in wine fermentations can be enumerated in the presence of high levels of *Saccharomyces* sp. For example, Gonzalez, et al. (2006) detected populations of AAB as low as 10 CFU per mL in the presence of overwhelming amounts of *Saccharomyces* (10^7 CFU per mL). By comparison, other survey methods such as PCR-DGGE, or even microscopy, generally require at least 1,000 to 10,000 organisms per mL (Cocolin, et al. 2000). QPCR provides a rapid method of detection, compared to conventional plating methods. Organisms such as *D. bruxellensis* can be detected and enumerated in as little as one to two hours, which is a substantial improvement on the five to 10 days required for conventional analysis by plates (Phister and Mills 2003). This time difference provides winemakers the opportunity to intervene long before spoilage is an issue.

The targets for QPCR assays vary between organisms; the most common is an rRNA gene in each organism, as this may be the only sequence information available for many wine-related organisms (Neeley, et al. 2005; Gonzalez, et al. 2006; Rawsthorne and Phister 2006). However, other sequences have been targeted, such as the gene encoding of the malolactic enzyme from *O. oeni* (Pinzani, et al. 2004), or even bands isolated by RAPD-PCR from *S. cerevisiae* (Martorell, et al. 2005). In a most prescient example, Delaherche, et al. (2004) used QPCR to solely enumerate exopolysaccharide-producing, or “ropy,” strains of *Pediococcus* in wine. Production of exopolysaccharide by pediococci in wine can result in a viscous, unpalatable spoilage. By targeting the *dps* gene within a pediococcal exopolysaccharide cluster (Walling, et al., 2005), the authors were able to directly enumerate only those strains that had potential for ropy spoilage. This is a promising addition to the QPCR approach because in many cases the pediococci present might not harbor the specific genes responsible for a taint. In such a situation QPCR-based enumeration of microbes at the species level will not determine the potential risk for a certain taint.

At present the promise of QPCR approaches as a means to more fully describe wine microbial ecology is yet unrealized. While a large number of QPCR detection assays have been developed, few have used these approaches in larger scale ecological studies. Regardless, since the approach is now commonly used in a number of service laboratories for microbial screening of wines, one would predict that direct QPCR-based survey data on different wineries' microbiota will be forthcoming.

3 Culture-dependent Studies on Wine Microbial Ecology

Given its prominence in various countries, it is not surprising that wine fermentations and winery environments are some of the most studied microbial ecologies. Indeed, the microbial diversity present in wine production, described in a previous section, was obtained chiefly through enrichment studies. Unlike the use of culture-independent approaches that revealed metabolically active, but

non-culturable, populations in wine (Millet and Lonvaud-Funel 2000), the main benefit of new molecular identification methods of enriched isolates is to further delineate the species (or subspecies) of yeast and bacteria present in different wine settings. This has provided new resolution to the types of yeasts and bacteria present but, unlike the culture-independent methods, has not dramatically changed the overall view of the ecology. This section will focus on select molecular identification methods used in post-enrichment analyses of wine-related yeast and bacteria that have advanced our understanding of wine microbial ecology. In general, these molecular methods fall into two categories: those that seek to identify genus and species (and/or subspecies), and those that seek to differentiate strains of the same species. For general information on standard enrichment methods used for wine-related yeasts or bacteria readers are directed elsewhere (Boulton, et al. 1996; Deák and Beuchat 1996; Fugelsang 1997; Boundy-Mills 2006).

3.1 Species and Subspecies Identification and Differentiation

3.1.1 rRNA Gene Sequence Analysis

Clearly no advance in microbial identification has had a more significant impact on rapid identification of enrichment isolates than ribosomal RNA gene analysis (Olsen, et al. 1994). Numerous methods have been developed to profile and catalog differences among rRNA genes in both yeast and bacteria (Towner and Cockayne 1993; Fernandez-Espinar, et al. 2006). Perhaps the most significant for identification of genus and species is the direct sequencing of the 16S rRNA gene in bacteria (Cole, et al. 2005) and the 26S rRNA gene (Kurtzman and Robnett 1998), and, to a lesser extent, the 18S rRNA gene (Valente, et al. 1999) in yeast through comparison to existing databases. Indeed, these methods combined with advances in colony PCR methodology (Hofmann and Brian 1991; Ward 1992) have enabled rapid identification of species from microbial isolates enriched from wine. A common approach is to segregate isolates on the basis of colony morphology and identify genus and species by rRNA gene-sequencing from only select morphotypes. A drawback to sequencing methods is the cost of sequencing which, while decreasing, is still prohibitive for large-scale ecological studies.

In addition to the rRNA gene sequences themselves, the spacer regions between the rRNA genes in yeast (Montrocher, et al. 1998; Egli and Henick-Kling 2001; Belloch, et al. 2002) and bacteria (Le Jeune and Lonvaud-Funel 1997) have been used to further differentiate both yeast and bacteria in wine, primarily assisting in subspecies differentiation.

3.1.2 rRNA Gene RFLP Approaches

An economical method to identify genus and species of yeasts enriched from wine fermentations is the ITS-restriction fragment length polymorphism (RFLP) method

(Guillamon, et al. 1998; Esteve-Zarzoso, et al. 1999). To facilitate identification of yeasts these authors developed a web-accessible database of yeast ITS RFLP patterns enabling direct comparisons of newly generated ITS RFLP patterns (<http://www.yeast-id.com/english/index.html>). While this approach is not as discriminatory as 26S rRNA gene sequence (Arias, et al. 2002), the method has been used to profile yeast species evolution in numerous wine settings (Fernandez, et al. 1999; Granchi, et al. 1999; Pramateftaki, et al. 2000; Esteve-Zarzoso, et al. 2001; Jemec, et al. 2001; Raspor, et al. 2002).

Similar rRNA gene RFLP approaches have been used to identify wine-related bacteria. RFLP of amplified 16S rRNA gene has been employed for identification of wine-related LAB (Rodas, et al. 2003) and AAB (Poblet, et al. 2000). Additional species level discrimination has been achieved by RFLP of the 16S-23S intergenic spacer region (Ruiz, et al. 2000). Since these rRNA gene RFLP approaches require a database of RFLP patterns with which to identify new strains, they are infrequently used by comparison to partial sequencing of 16S rRNA gene as a means to speciate wine-related bacteria.

3.1.3 PCR-DGGE

Others have employed PCR-DGGE or PCR-TGGE of rRNA gene segments to differentiate individual wine yeast isolates (Hernan-Gomez, et al. 2000; Manzano, et al. 2004; Manzano, et al. 2005; Manzano, et al. 2006). Given the discriminatory power of T/DGGE this method works well as long as standards are run for each potential species that one might observe in that environmental niche. This approach is also useful to monitor primary enrichment cultures from wine or grape substrates. Bae and co-workers (Bae, et al. 2006) used this approach to reveal the LAB on grape surfaces that were enriched via different media. In general, however, T/DGGE approaches for identification purposes are technically problematic since control strains must be present in the gel and band co-migration with known standards is not clear confirmation of identify.

3.1.4 Probes

A less popular approach to identify wine microbes enriched on various media is use of specific nucleotide probes targeting ribosomal RNA genes. Stender, et al. (2001) used peptide nucleic acid probes to identify the spoilage yeast, *D. bruxellensis*, on enrichment plates. Recently Xufre, et al. (2006) developed 26S rRNA gene probes for identification of numerous wine-related yeast including *S. cerevisiae*, *C. stellata*, *H. uvarum*, *H. guilliermondii*, *K. thermotolerans*, *K. marxianus*, *T. delbrueckii*, *Pi. membranaefaciens* and *Pi. anomala*. While these latter probes were fluorescently labeled for use in direct *in situ* hybridization applications, the authors have only demonstrated their utility in identifying yeast colonies post-enrichment. Others have used more specific genomic probes to differentiate colonies of closely related LAB species (Sohier, et al. 1999).

3.1.5 PCR Screens for Taint-related Genes within Isolates

Linkage of specific genes within wine microbes to specific taints has led to a series of post-enrichment screens to understand the ecological distribution of these alleles. For the most part this effort has focused on wine LAB as few taint-related genes have been cloned and characterized from spoilage yeasts or AAB. In the recent past genes responsible for ropiness (Gindreau, et al. 2001), acrolein taint (Claisse and Lonvaud-Funel 2001) and biogenic amine production (Le Jeune, et al. 1995; Landete, et al. 2005; Costantini, et al. 2006), have become targets for PCR screens.

3.2 *Methods for Intraspecific Differentiation*

While rRNA gene analysis has fostered a monumental advance in wine microbiology by enabling rapid speciation of isolates, the tools for differentiating between different strains of the same species have advanced as well. This, in turn, has enabled a much more exquisite dissection of individual constituents at the strain level within the winery and local environs. Two general approaches have been taken to differentiate subspecies and strains of wine-related bacteria or yeast. The first employs whole or sub-genomic analysis through pulse field gel electrophoresis (PFGE), various genomic RFLP methods or newer array approaches to directly examine the isolate genomic makeup. The second approach employs whole genome PCR sampling techniques that result in strain-specific fingerprints from which differentiation of strains is possible.

3.2.1 Wine Yeast

Until recently the two main “gold standard” methods for differentiation of wine yeast strains have been whole genome PFGE (karyotyping [Carle and Olson 1985]) or RFLP of the mitochondrial genome (mito-RFLP [Lee, et al. 1985]). Because of its easier application, more researchers have employed the mito-RFLP method to differentiate yeast strains within wine fermentations revealing similar successions of different *S. cerevisiae* strains throughout various wine fermentations (Querol, et al. 1994; Sabate, et al. 1998).

Other groups have developed whole genome PCR sampling approaches that amplify targeted or arbitrary segments of the yeast genome resulting in strain-specific fingerprints. In general whole genome PCR sampling techniques are popular because of the ease of use, however, the reproducibility of these approaches – which vary among laboratories, personnel and thermocyclers – is often problematic. One approach employs primers to amplify repeated regions in the genome such as delta elements of the Ty transposon (Ness, et al. 1993), intron splice sites (de Barros Lopes, et al. 1998), minisatellites (Mannazzu, et al. 2002; Marinangeli, et al. 2004) or micro-satellite markers (Hennequin, et al. 2001). Another approach uses primers

that do not target known repeated sequences, but work by priming at arbitrary sites to generate a specific fingerprint enabling differentiation of yeast strains (random amplified polymorphic DNA [RAPD]) (Baleiras Couto, et al. 1996; Torriani, et al. 1999; Xufre, et al. 2000; Bujdoso, et al. 2001), REP (Hierro, et al. 2004) and ERIC (Hierro, et al. 2004). A modification of these approaches, amplified fragment length polymorphism (AFLP), employs a chromosomal shearing step prior to adapter ligation and PCR using adapter-based primers. De Barros Lopes, et al. (1999) used this approach to differentiate strains of *S. cerevisiae* and *D. bruxellensis*.

In the last 15 years these methods have tremendously enabled strain discrimination emerging from plating studies on wine and facilitated a multitude of studies. Most have examined *S. cerevisiae* populations in different wine settings. Schutz and Gafner (1993) employed karyotyping to demonstrate the diversity of *S. cerevisiae* strains in spontaneous wine fermentations, compared to inoculated fermentations. The spontaneous fermentations were shown to contain several different strains of *S. cerevisiae* that competed within the fermentation, while the inoculated fermentation was dominated by the inoculated strain (Schutz and Gafner 1994). Around the same time, Querol and co-workers (1992, 1994) employed the mito-RFLP approach to characterize spontaneous and inoculated fermentations and noted similar results. Many subsequent studies have since revealed a multitude of *S. cerevisiae* strains present in spontaneous and inoculated fermentations in various regions or oenological conditions (Gutierrez, et al. 1997; Epifanio, et al. 1999; Esteve-Zarzoso, et al. 2001; Granchi, et al. 2003; Torija, et al. 2003; Demuyter, et al. 2004; Blanco, et al. 2006; Lopes, et al. 2006). Interestingly *S. cerevisiae* strains that dominated fermentations in one year, either through inoculation or emerging indigenously, have been shown to dominate the same winery in the following year (Constanti, et al. 1997; Sabate, et al. 1998).

Perhaps the most promising advance to intraspecific discrimination of wine yeasts has been achieved through use of whole genome sequences. Full genome sequences are available for *S. cerevisiae* (Goffeau, et al. 1996), *Schizosaccharomyces pombe* (Wood, et al. 2002), *K. lactis* (Dujon, et al. 2004) and *Db. hansenii* (Dujon, et al. 2004). In addition several genome sequencing projects for wine-related yeasts or fungi are currently underway including *D. bruxellensis*, *K. thermotolerans*, *S. bayanus* and *B. cinerea* (see <http://www.ncbi.nlm.nih.gov>). An increasingly common approach to examine strain evolution and differentiation is to use comparative genomic hybridization (CGH) with whole or partial genomic arrays. In this fashion chromosomal loci that are shared or missing among strains are documented with the level of discrimination dictated by the level of genome coverage present on the array. Moreover, both small nucleotide polymorphisms and/or larger gene deletions can be witnessed. More importantly, the biological implications of these polymorphisms can be inferred by the encoded genetic content. As a consequence, array-based differentiation is fundamentally more informative than the fingerprinting methods described above, given that the witnessed differences are linked to an *in silico* prediction of the underlying metabolism. To date, relatively few groups have used CGH to characterize wine yeasts. Winzeler and co-workers (2003) characterized

14 different *S. cerevisiae* strains, including several wine-related isolates from Tuscany. They noted a bias for polymorphisms, both gene deletions and single nucleotide changes, in subtelomeric regions. Moreover a genealogical relationship of the 14 strains was developed on the basis of 11,115 probes clearly demonstrated the phylogenetic clustering of the “wild” wine-related strains and separation from the other laboratory strains. Dunn and co-workers (2005) used a similar approach to characterize four different commercial *S. cerevisiae* strains used in winemaking. The four strains showed similar differences from the sequenced *S. cerevisiae* strain S288C (a laboratory strain) and revealed a moderate level of inter-strain differences mostly in transporter genes. These differences were documented functionally in that strains with lower numbers of metallothionien alleles (CUP1) were shown to be sensitive to the fungicide, sulfomethuron methyl.

3.2.2 Wine Bacteria

Similar to the situation with wine yeast, a prominent and reproducible method for bacterial strain differentiation is through use of PFGE, in this case to separate genomic RFLP patterns generated by using rare cutter restriction enzymes (Kelly, et al. 1993). Often whole genome PCR sampling approaches, such as RAPDs, are used in concert with PFGE and rRNA gene typing to provide a polyphasic description of isolates (Rodas, et al. 2005).

A major focus of the wine bacterial analysis has been the malolactic starter culture, *O. oeni*. Studies using a variety of molecular approaches to discriminate isolates have suggested a homogeneous nature to the species (Kelly, et al. 1993; Viti, et al. 1996; Zavaleta, et al. 1997; Sato, et al. 2001). Using PFGE methods, Tenreiro and co-workers (1994) proposed two major lineages for *O. oeni*. However, a subsequent analysis suggested the two groupings were less divergent than originally believed (Ze-Ze, et al. 2000). Recently De Rivas, et al. (2004) used multi-locus sequence typing (MLST) of five genes (*gyrB*, *ddl*, *mleA*, *pgm* and *recP*) to examine allelic diversity and population structure of various oenococcal isolates. Interestingly, MLST was able to differentiate 18 strains that could only be differentiated into two groups by ribotyping. This allelic diversity suggests a higher level of genetic heterogeneity among oenococcal isolates than had been previously suggested by other molecular typing methods. De Rivas, et al. (2004) also concluded that recombination has played a major role in generating genetic diversity in *O. oeni*. Interestingly, the same authors recently characterized 16 *Lb. plantarum* strains using MLST and arrived at much the same general conclusion: the strains possessed a high level of sequence heterozygosity that suggested frequent recombination (de las Rivas, et al. 2006).

The recent publication of whole genome sequences for various wine-related LAB (Kleerebezem, et al. 2003; Makarova, et al. 2006) and AAB (Prust, et al. 2005), has laid the foundation for future intra-specific discrimination. To date, however, only one CGH study has been published on a wine-related species, *Lb. plantarum* (Molenaar, et al. 2005), albeit none of the strains in that work came from

the wine environment. Regardless, most of the variability noted centered on genes involved in sugar utilization, in addition to bacteriocin, exopolysaccharide and prophage encoded differences. From this work, the *Lb. plantarum* clade was delineated into two distinguishable clusters, a conclusion confirming previous molecular differentiation studies.

4 Future Directions for Wine Fermentation Ecology

As advances in molecular methods improve it is clear that the ability to discriminate specific strains, as well as to define their impact on wine production, will improve. One advance that is readily accessible, and decreasing in cost, is the use of high throughput QPCR assays. As this technology becomes more accessible and moves into the average winery laboratory, the ability to rapidly profile and enumerate the microbial contents of winery fermentations could become a normal part of wine-making. With such information in hand winemakers could more readily (and rapidly) spot problem microbes, or even problem alleles, within their fermentations. If such data were collected in a winery each year, winemakers could look to historical data on the previous year's fermentations to help investigate specific anomalies. From a more academic perspective the high throughput QPCR assays are ideal for doing "epidemiological" surveys to identify the reservoirs of spoilage microbes such as *Br. bruxellensis*, or even problem alleles, such as histidine decarboxylases from LAB. The latter approach is currently underway in several laboratories and will provide insight into the ecological reservoirs of genes associated with specific wine taints.

Similar to QPCR, a greater accessibility of microarrays will strongly influence ecological research in wine. Arrays have been created which contain specific 16S rRNA gene sequences to allow discrimination of large number of microbial clades within a single hybridization event (Wilson, et al. 2002). Such arrays allow simultaneous detection and discrimination of diverse sets of microbes and, with high throughput methods, would enable comprehensive microbial ecological analysis (Zhou 2003; Gentry, et al. 2006). To date, however, no such arrays containing specific 26S rRNA gene sequences exist for assessing yeast diversity. Regardless, as the cost of the arrays decrease and utility of the technology advances, these tools will undoubtedly provide wine researchers with methods to advance our understanding of the microbial changes inherent in wine production.

It is hard to discuss the future directions of wine microbiological research without commenting on the tremendous advances in DNA sequencing technology. The exponential increase in publicly available microbial genome sequences will continue to induce wine researchers to adapt to this new bioinformatic landscape. While *S. cerevisiae* was the first eukaryote sequenced over 10 years ago (Goffeau, et al. 1996), we are still in the early stages of genomic analysis of the many different microorganisms that impact wine fermentations. Many more genome sequences are needed. In particular access to sequences for a large number of *S. cerevisiae* and

O. oeni strains currently employed as starter cultures would advance our understanding of the encoded metabolic diversity present within these important species, and help optimize their application in wine production. In addition, genome sequence for a number of other yeasts (e.g., *H. uvarum*, and *C. stellata*), AAB (e.g., *A. aceti*, *A. pasteurianus*, *G. hanseii*) and LAB (e.g., *Lb. hilgardii*, *Lb. buchneri*, *P. damnosus*, *P. parvulus*) species would enable new insights into these important species. Inherent in that new information will be a profoundly better ability to differentiate additional strains and a new understanding of their role in wine production and wine flavor development.

Given that different wine production schemes and regional styles foster different microbial consortia, it is not unreasonable to assume a sort of microbial “terroir” which, in part, endows specific wineries and winemaking styles with a particular flavor profile. However, fully viewing the underlying microbial changes inherent in such environments is problematic. The advent of inexpensive DNA sequencing technologies has now fostered a new approach, metagenomic sequencing, in which all DNA present in a particular environmental niche are cloned and sequenced. Such approaches are now frequently used to profile both the microbial diversity, and also the encoded metabolic capacity from environments (Tyson, et al. 2004). From such an approach it is easy to imagine how a robust description of the microbial community in a specific wine fermentation could be revealed. Moreover the resultant sequence description would reveal the underlying genetic potential within such a fermentation, enabling *in silico* metabolic reconstructions and comparisons (e.g., temporal changes within a fermentation or comparison between fermentations), all without considering microbial cell wall boundaries. In essence the aggregate microbiota of the wine fermentation itself could be considered a “super-organism” with defined metabolic capacity. It is from these types of approaches that *in silico* metabolic models can be generated from complex ecosystems. With such models in hand, future researchers—and winemakers—will have an abundance of information to ensure more flavorful and consistent fermentations in the years to come.

References

- Adams, M. R. 1998. Vinegar, p. 1–44. In Wood, B. J. B. (ed.), *Microbiology of Fermented Foods*, 2 ed., Blackie Academic & Professional, London, UK.
- Alexandre, H., and C. Charpentier. 1998. Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Ind. Microbiol. Biotechnol.* 20:20–27.
- Amerine, M. A., and R. E. Kunkee. 1968. Microbiology of winemaking. *Ann. Rev. Microbiol.* 22:323–58.
- Arias, C. R., J. K. Burns, L. M. Friedrich, R. M. Goodrich, and M. E. Parish. 2002. Yeast species associated with orange juice: evaluation of different identification methods. *Appl. Environ. Microbiol.* 68:1955–61.
- Bae, S., G. H. Fleet, and G. M. Heard. 2006. Lactic acid bacteria associated with wine grapes from several Australian vineyards. *J. Appl. Microbiol.* 100:712–27.
- Baleiras Couto, M. M., B. Eijmsa, H. Hofstra, J. H. Huis in't Veld, and J. M. van der Vossen. 1996. Evaluation of molecular typing techniques to assign genetic diversity among *Saccharomyces cerevisiae* strains. *Appl. Environ. Microbiol.* 62:41–6.

- Barbe, J. C., G. De Revel, A. Joyeux, A. Bertrand, and A. Lonvaud-Funel. 2001. Role of botrytized grape microorganisms in SO₂ binding phenomena. *J. Appl. Microbiol.* 90:34–42.
- Barer, M. R., and C. R. Harwood. 1999. Bacterial viability and culturability. *Adv. Microb. Physiol.* 41:93–137.
- Barnett, J. A., M. A. Delaney, E. Jones, A. B. Magson, and B. Winch. 1972. The numbers of yeasts associated with wine grapes of Bordeaux. *Arch. Microbiol.* 83:52–55.
- Bartowsky, E. J., and P. A. Henschke. 1999. Use of a polymerase chain reaction for specific detection of the malolactic fermentation bacterium *Oenococcus oeni* (formerly *Leuconostoc oenos*) in grape juice and wine sample. *Aust. J. Grape Wine Res.* 5:39–44.
- Bartowsky, E. J., D. Xia, R. L. Gibson, G. H. Fleet, and P. A. Henschke. 2003. Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* 36:307–14.
- Belloch, C., T. Fernandez-Espinar, A. Querol, M. Dolores Garcia, and E. Barrio. 2002. An analysis of inter- and intraspecific genetic variabilities in the *Kluyveromyces marxianus* group of yeast species for the reconsideration of the *K. lactis* taxon. *Yeast* 19:257–68.
- Bisson, L. F. 1999. Stuck and sluggish fermentations. *Amer. J. Enol. Vitic.* 50:107–119.
- Blanco, P., A. Ramilo, M. Cerdeira, and I. Orriols. 2006. Genetic diversity of wine *Saccharomyces cerevisiae* strains in an experimental winery from Galicia (NW Spain). *Ant. van Leeuwenhoek* 89:351–7.
- Blasco, L., S. Ferrer, and I. Pardo. 2003. Development of specific fluorescent oligonucleotide probes for *in situ* identification of wine lactic acid bacteria. *FEMS Microbiol. Lett.* 225:115–23.
- Borzani, W., and M. L. Vairo. 1958. Quantitative adsorption of methylene blue by dead yeast cells. *J. Bacteriol.* 76:251–5.
- Boulton, R. B., V. L. Singleton, L. F. Bisson, and R. E. Kunkee. 1996. Principles and practices of winemaking. Chapman & Hall, New York.
- Boundy-Mills, K. 2006. Methods for investigating yeast biodiversity. In Rosa, C. A., Peter, G. (eds.), Biodiversity and Ecophysiology of Yeasts, Springer Berlin.
- Boyd, A. R., T. S. Gunasekera, P. V. Attfield, K. Simic, S. F. Vincent, and D. A. Veal. 2003. A flow-cytometric method for determination of yeast viability and cell number in a brewery. *FEMS Yeast Res.* 3:11–6.
- Bujdoso, G., C. M. Egli, and T. Henick-Kling. 2001. Characterization of *Hanseniaspora* (Kloeckera) strains isolated in Finger Lakes wineries using physiological and molecular techniques. *Food Technol. Biotechnol.* 39:83–91.
- Carle, G. F., and M. V. Olson. 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci.* 82:3756–60.
- Casey, G. D., and A. D. Dobson. 2004. Potential of using real-time PCR-based detection of spoilage yeast in fruit juice—a preliminary study. *Int. J. Food Microbiol.* 91:327–35.
- Chaney, D., S. Rodriguez, K. Fugelsang, and R. Thornton. 2006. Managing high-density commercial scale wine fermentations. *J. Appl. Microbiol.* 100:689–98.
- Chatonnet, P., D. Dubourdieu, and J. N. Boidron. 1995. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. *Amer. J. Enol. Vitic.* 46:463–468.
- Ciani, M., and F. Maccarelli. 1998. Oenological properties of non-*Saccharomyces* yeasts associated with winemaking. *World J. Microbiol. Biotechnol.* 14:199–203.
- Claissé, O., and A. Lonvaud-Funel. 2001. Primers and a specific DNA probe for detecting lactic acid bacteria producing 3-hydroxypropionaldehyde from glycerol in spoiled ciders. *J. Food Prot.* 64:833–7.
- Cocolin, L., L. F. Bisson, and D. A. Mills. 2000. Direct profiling of the yeast dynamics in wine fermentations. *FEMS Microbiol. Lett.* 189:81–7.
- Cocolin, L., A. Heisey, and D. A. Mills. 2001. Direct identification of the indigenous yeasts in commercial wine fermentations. *Amer. J. Enol. Vitic.* 52:49–53.
- Cocolin, L., M. Manzano, S. Rebecca, and G. Comi. 2002. Monitoring of yeast population changes during continuous wine fermentation by molecular methods. *Amer. J. Enol. Vitic.* 53:24–27.

- Cocolin, L., and D. A. Mills. 2003. Wine yeast inhibition by sulfur dioxide: a comparison of culture-dependent and -independent methods. *Amer. J. Enol. Vitic.* 54:125–130.
- Cocolin, L., K. Rantsiou, L. Iacumin, R. Zironi, and G. Comi. 2004. Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkera anomalous* in spoiled wines. *Appl. Environ. Microbiol.* 70:1347–55.
- Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje. 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33:D294–6.
- Constanti, M., M. Poblet, L. Arola, A. Mas, and J. M. Guillamon. 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Amer. J. Enol. Vitic.* 48:339–344.
- Constanti, M., C. Reguant, M. Poblet, F. Zamora, A. Mas, and J. M. Guillamon. 1998. Molecular analysis of yeast population dynamics: Effect of sulphur dioxide and inoculum on must fermentation. *Int. J. Food Microbiol.* 41:169–175.
- Costantini, A., M. Cersosimo, V. Del Prete, and E. Garcia-Moruno. 2006. Production of biogenic amines by lactic acid bacteria: screening by PCR, thin-layer chromatography, and high-performance liquid chromatography of strains isolated from wine and must. *J. Food Prot.* 69:391–6.
- Coton, E., G. Rollan, A. Bertrand, and A. Lonvaud-Funel. 1998. Histamine-producing lactic acid bacteria in wines: Early detection, frequency, and distribution. *Amer. J. Enol. Vitic.* 49:199–204.
- Dahllof, I., H. Baillie, and S. Kjelleberg. 2000. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Appl. Environ. Microbiol.* 66:3376–80.
- Davenport, R. R. 1974. Microecology of yeasts and yeast-like organisms associated with an English vineyard. *Vitis* 13:123–130.
- Davidson, P. M. 1997. Chemical preservatives and natural antimicrobial compounds, In Doyle, M., Beuchat, L. R., and Montville, T. (eds.), *Food Microbiology Fundamentals and Frontiers*. ASM Press, Washington DC, p. 520–556.
- Davis, C. R., D. Wibowo, R. Eschenbruch, T. H. Lee, and G. H. Fleet. 1985. Practical implications of malolactic fermentation: A review. *Amer. J. Enol. Vitic.* 36:290–301.
- de Barros Lopes, M., S. Rainieri, P. A. Henschke, and P. Langridge. 1999. AFLP fingerprinting for analysis of yeast genetic variation. *Int. J. Syst. Bacteriol.* 49:915–24.
- de Barros Lopes, M., A. Soden, A. L. Martens, P. A. Henschke, and P. Langridge. 1998. Differentiation and species identification of yeasts using PCR. *Int. J. Syst. Bacteriol.* 48:279–86.
- de Boer, E., and R. R. Beumer. 1999. Methodology for detection and typing of food-borne microorganisms. *Int. J. Food Microbiol.* 50:119–30.
- de Las Rivas, B., A. Marcobal, and R. Munoz. 2004. Allelic diversity and population structure in *Oenococcus oeni* as determined from sequence analysis of housekeeping genes. *Appl. Environ. Microbiol.* 70:7210–9.
- de Las Rivas, B., A. Marcobal, and R. Munoz. 2006. Development of a multi-locus sequence typing method for analysis of *Lactobacillus plantarum* strains. *Microbiology* 152:85–93.
- Deák, T., and L. R. Beuchat. 1996. *Handbook of food spoilage yeasts*. CRC Press, Boca Raton.
- Delaherche, A., O. Claisse, and A. Lonvaud-Funel. 2004. Detection and quantification of *Brettanomyces bruxellensis* and 'ropy' *Pediococcus damnosus* strains in wine by real-time polymerase chain reaction. *J. Appl. Microbiol.* 97:910–5.
- Delfini, C., and J. V. Formica. 2001. *Wine microbiology : Science and technology*. Marcel Dekker, New York.
- Demuyter, C., M. Lollier, J. L. Legras, and C. Le Jeune. 2004. Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation, for three consecutive years, in an Alsatian winery. *J. Appl. Microbiol.* 97:1140–8.
- Dent, K. C., J. R. Stephen, and W. E. Finch-Savage. 2004. Molecular profiling of microbial communities associated with seeds of *Beta vulgaris* subsp. *vulgaris* (sugar beet). *J. Microbiol. Methods*. 56:17–26.

- Dittrich, H. H. 1991. Wine and Brandy, In Rehm, H.-J. and Reed, G. (eds.), *Biotechnology: A multi-volume comprehensive treatise*, Weinheim New York, p. 463–506.
- Divol, B., and A. Lonvaud-Funel. 2005. Evidence for viable but non-culturable yeasts in botrytis-affected wine. *J. Appl. Microbiol.* 99:85–93.
- du Toit, W. J., and M. G. Lambrechts. 2002. The enumeration and identification of acetic acid bacteria from South African red wine fermentations. *Int. J. Food Microbiol.* 74:57–64.
- du Toit, W. J., I. S. Pretorius, and A. Lonvaud-Funel. 2005. The effect of sulphur dioxide and oxygen on the viability and culturability of a strain of *Acetobacter pasteurianus* and a strain of *Brettanomyces bruxellensis* isolated from wine. *J. Appl. Microbiol.* 98:862–71.
- Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. de Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J.-M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. de Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J.-M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G.-F. Richard, M.-L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J.-L. Souciet. 2004. Genome evolution in yeasts. *Nature* 430:35–44.
- Dunn, B., R. P. Levine, and G. Sherlock. 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics* 6:53.
- Egli, C. M., W. D. Efinger, C. M. Mittrakul, and T. Henick-Kling. 1998. Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. *J. Appl. Microbiol.* 85:779–789.
- Egli, C. M., and T. Henick-Kling. 2001. Identification of *Brettanomyces/Dekkera* species based on polymorphism in the rRNA internal transcribed spacer region. *Amer. J. Enol. Vitic.* 52:241–247.
- Epifanio, S., A. R. Gutierrez, M. P. Santamaria, and R. Lopez. 1999. The influence of enological practices on the selection of wild yeast strains in spontaneous fermentation. *Amer. J. Enol. Vitic.* 50:219–224.
- Esteve-Zarzoso, B., C. Belloch, F. Uruburu, and A. Querol. 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol.* 49:329–337.
- Esteve-Zarzoso, B., M. J. Peris-Toran, E. Garcia-Maiquez, F. Uruburu, and A. Querol. 2001. Yeast population dynamics during the fermentation and biological aging of sherry wines. *Appl. Environ. Microbiol.* 67:2056–61.
- Fernandez-Espinar, M. T., P. Martorell, R. de Llanos, and A. Querol. 2006. Molecular methods to identify and characterize yeasts in foods and beverages, In Querol, A. and Fleet, G. H. (eds.), *Yeasts in Food and Beverages*. Springer, Berlin Heidelberg, p. 55–82.
- Fernandez, M. T., J. F. Ubeda, and A. I. Briones. 1999. Comparative study of non-*Saccharomyces* microflora of musts in fermentation, by physiological and molecular methods. *FEMS Microbiol. Lett.* 173:223–229.
- Fleet, G. H. 1990. Food spoilage yeasts. In Spencer, J. and Spencer, D. (ed.), *Yeast Technology*. Springer-Verlag, New York, p. 124–166.
- Fleet, G. H. 1998. The microbiology of alcoholic beverages. In *Microbiology of Fermented Foods*, 2nd ed. Blackie Academic & Professional, New York, p. 217–262.
- Fleet, G. H. 2003. Yeast interactions and wine flavor. *Int. J. Food Microbiol.* 86:11–22.
- Fleet, G. H., and M. A. Mian. 1998. Induction and repair of sublethal injury in food spoilage yeasts. *J. Food Mycol.* 1:85–93.
- Fugelsang, K. 1997. *Wine Microbiology*. Chapman and Hall, New York, NY.
- Fugelsang, K. C., and B. W. Zoecklein. 2003. Population dynamics and effects of *Brettanomyces bruxellensis* strains on pinot noir (*Vitis vinifera* L.) wines. *Amer. J. Enol. Vitic.* 54:294–300.

- Fuglsang, C. C., C. Johansen, S. Christgau, and J. Adler Nissen. 1995. Antimicrobial enzymes: Applications and future potential in the food industry. *Trends Food Sci. Technol.* 6:390–396.
- Gao, C., and G. H. Fleet. 1988. The effects of temperature and pH on the ethanol tolerance of the wine yeasts, *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. *J. Appl. Bacteriol.* 65:405–410.
- Gentry, T. J., G. S. Wickham, C. W. Schadt, Z. He, and J. Zhou. 2006. Microarray applications in microbial ecology research. *Microb. Ecol.* 52:159–75.
- Gindreau, E., E. Walling, and A. Lonvaud-Funel. 2001. Direct polymerase chain reaction detection of rosy *Pediococcus damnosus* strains in wine. *J. Appl. Microbiol.* 90:535–542.
- Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver. 1996. Life with 6,000 genes. *Science* 274: 563–7.
- Gonzalez, A., N. Hierro, M. Poblet, A. Mas, and J. M. Guillamon. 2006. Enumeration and detection of acetic acid bacteria by real-time PCR and nested PCR. *FEMS Microbiol. Lett.* 254:123–8.
- Graca da Silveira, M., M. Vitoria San Romao, M. C. Loureiro-Dias, F. M. Rombouts, and T. Abee. 2002. Flow cytometric assessment of membrane integrity of ethanol-stressed *Oenococcus oeni* cells. *Appl. Environ. Microbiol.* 68:6087–93.
- Gracias, K. S., and J. L. McKillip. 2004. A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Can. J. Microbiol.* 50:883–90.
- Granchi, L., M. Bosco, A. Messini, and M. Vincenzini. 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. *J. Appl. Microbiol.* 87:949–956.
- Granchi, L., D. Ganucci, C. Viti, L. Giovannetti, and M. Vincenzini. 2003. *Saccharomyces cerevisiae* biodiversity in spontaneous commercial fermentations of grape musts with ‘adequate’ and ‘inadequate’ assimilable-nitrogen content. *Lett. Appl. Microbiol.* 36:54–8.
- Guillamon, J. M., J. Sabate, E. Barrio, J. Cano, and A. Querol. 1998. Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch. Microbiol.* 169:387–92.
- Gutierrez, A. R., R. Lopez, M. P. Santamaria, and M. J. Sevilla. 1997. Ecology of inoculated and spontaneous fermentations in Rioja (Spain) musts, examined by mitochondrial DNA restriction analysis. *Int. J. Food Microbiol.* 36:241–5.
- Hanna, S. E., C. J. Connor, and H. H. Wang. 2005. Real-time polymerase chain reaction for the food microbiologist: Technologies, applications, and limitations. *J. Food Sci.* 70:49–53.
- Head, I. M., J. R. Saunders, and R. W. Pickup. 1998. Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35:1–21.
- Hennequin, C., A. Thierry, G. F. Richard, G. Lecointre, H. V. Nguyen, C. Gaillardin, and B. Dujon. 2001. Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. *J. Clin. Microbiol.* 39:551–9.
- Hernan-Gomez, S., J. C. Espinosa, and J. F. Ubeda. 2000. Characterization of wine yeasts by temperature gradient gel electrophoresis (TGGE). *FEMS Microbiol. Lett.* 193:45–50.
- Herrero, M., C. Quiros, L. A. Garcia, and M. Diaz. 2006. Use of flow cytometry to follow the physiological states of microorganisms in cider fermentation processes. *Appl. Environ. Microbiol.* 72:6725–33.
- Hierro, N., B. Esteve-Zarzoso, A. Gonzalez, A. Mas, and J. M. Guillamon. 2006. Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Appl. Environ. Microbiol.* 72:7148–55.
- Hierro, N., A. Gonzalez, A. Mas, and J. M. Guillamon. 2004. New PCR-based methods for yeast identification. *J. Appl. Microbiol.* 97:792–801.
- Hofmann. 1968. Biochemical changes caused by *Botrytis cinerea* and *Rhizopus nigricans* in grape must. *S. African J. Agric. Sci.* 11:335–348.
- Hofmann, M. A., and D. A. Brian. 1991. Sequencing PCR DNA amplified directly from a bacterial colony. *Biotechniques* 11:30–1.

- Ibeas, J. I., I. Lozano, F. Perdignes, and J. Jimenez. 1996. Detection of *Dekkera-Brettanomyces* strains in sherry by a nested PCR method. *Appl. Environ. Microbiol.* 62:998–1003.
- Jemec, K. P., N. Cadez, T. Zagorc, V. Bubic, A. Zupec, and P. Raspor. 2001. Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiol.* 18:247–259.
- Joyeux, A., S. Lafonlaurcade, and P. Ribereaugayon. 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. *Appl. Environ. Microbiol.* 48:153–156.
- Kell, D. B., A. S. Kaprelyants, D. H. Weichart, C. R. Harwood, and M. R. Barer. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Ant. van Leeuwenhoek* 73:169–87.
- Kelly, W. J., C. M. Huang, and R. V. Asmundson. 1993. Comparison of *Leuconostoc oenos* Strains by Pulsed-Field Gel Electrophoresis. *Appl. Environ. Microbiol.* 59:3969–3972.
- Kelly, W. J., C. M. Huang, and R. V. Asmundson. 1993. Comparison of *Leuconostoc oenos* strains by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* 59:3969–3972.
- Kerstens, K., P. Lisdianti, K. Komagata, and J. Swings. 2006. The Family Acetobacteraceae: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*. In Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrandt, E. (eds.), *The Prokaryotes*, Vol. 5. Springer, New York, p. 163–200.
- Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Turchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, and R. J. Siezen. 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci.* 100:1990–5.
- Kurtzman, C. P., and C. J. Robnett. 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Ant. van Leeuwenhoek* 73:331–71.
- Landete, J. M., S. Ferrer, and I. Pardo. 2005. Which lactic acid bacteria are responsible for histamine production in wine? *J. Appl. Microbiol.* 99:580–6.
- Le Jeune, C., A. Lonvaud-Funel, B. ten Brink, H. Hofstra, J. M. van der Vossen. 1995. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. *J. Appl. Bacteriol.* 78:316–26.
- Le Jeune, C., and A. Lonvaud-Funel. 1997. Sequence of DNA 16S/23S spacer region of *Leuconostoc oenos* (*Oenococcus oeni*): application to strain differentiation. *Res. Microbiol.* 148:79–86.
- Le-Roux, G., R. Eschenbruch, and S.-I. De-Bruin. 1973. The microbiology of South African winemaking. III. The microflora of healthy and *Botrytis cinerea* infected grapes. *Phytophylactica* 5:51–54.
- Lee, S. Y., F. B. Knudsen, and R. O. Poyton. 1985. Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. *J. Inst. Brew.* 91:169–173.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25:402–8.
- Lonvaud-Funel, A. 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. *Ant. van Leeuwenhoek* 76:317–31.
- Lopes, C. A., T. L. Lavalley, A. Querol, and A. C. Caballero. 2006. Combined use of killer biotype and mtDNA-RFLP patterns in a Patagonian wine *Saccharomyces cerevisiae* diversity study. *Ant. van Leeuwenhoek* 89:147–56.
- Lopez, I., F. Ruiz-Larrea, L. Cocolin, E. Orr, T. Phister, M. Marshall, J. VanderGheynst, and D. A. Mills. 2003. Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 69:6801–7.
- Lopez, V., M. T. Fernandez-Espinar, E. Barrio, D. Ramon, and A. Querol. 2003. A new PCR-based method for monitoring inoculated wine fermentations. *Int. J. Food Microbiol.* 81:63–71.
- Loureiro, V., and M. Malfeito-Ferreira. 2003. Spoilage yeasts in the wine industry. *Int. J. Food Microbiol.* 86:23–50.
- Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar, S. Lucas,

- K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes, Y. Goh, A. Benson, K. Baldwin, J. H. Lee, I. Diaz-Muniz, B. Dosti, V. Smeianov, W. Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H. Rawsthorne, D. Tamir, C. Parker, F. Breidt, J. Broadbent, R. Hutkins, D. O'Sullivan, J. Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, and D. Mills. 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci.* 103:15611–6.
- Malacrino, P., G. Zapparoli, S. Torriani, and F. Dellaglio. 2001. Rapid detection of viable yeasts and bacteria in wine by flow cytometry. *J. Microbiol. Methods* 45:127–34.
- Mannazzu, I., E. Simonetti, P. Marinangeli, E. Guerra, M. Budroni, M. Thangavelu, F. Clementi. 2002. SED1 gene length and sequence polymorphisms in feral strains of *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* 68:5437–5444.
- Manzano, M., L. Cocolin, L. Iacumin, C. Cantoni, and G. Comi. 2005. A PCR-TGGE (Temperature Gradient Gel Electrophoresis) technique to assess differentiation among enological *Saccharomyces cerevisiae* strains. *Int. J. Food Microbiol.* 101:333–9.
- Manzano, M., L. Cocolin, B. Longo, and G. Comi. 2004. PCR-DGGE differentiation of strains of *Saccharomyces sensu stricto*. *Ant. van Leeuwenhoek* 85:23–7.
- Manzano, M., D. Medrala, C. Giusto, I. Bartolomeoli, R. Urso, and G. Comi. 2006. Classical and molecular analyses to characterize commercial dry yeasts used in wine fermentations. *J. Appl. Microbiol.* 100:599–607.
- Marsh, T. L. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2:323–7.
- Marinangeli, P., D. Angelozzi, M. Ciani, F. Clementi and I. Mannazzu. 2004. Minisatellites in *Saccharomyces cerevisiae* genes encoding cell wall proteins: A new way towards wine strain characterisation. *FEMS Yeast Res.* 4: 427–435.
- Martorell, P., A. Querol, and M. T. Fernandez-Espinar. 2005. Rapid identification and enumeration of *Saccharomyces cerevisiae* cells in wine by real-time PCR. *Appl. Environ. Microbiol.* 71:6823–30.
- Millet, V., and A. Lonvaud-Funel. 2000. The viable but non-culturable state of wine microorganisms during storage. *Lett. Appl. Microbiol.* 30:136–41.
- Mills, D. A., E. A. Johannsen, and L. Cocolin. 2002. Yeast diversity and persistence in botrytis-affected wine fermentations. *Appl. Environ. Microbiol.* 68:4884–93.
- Molenaar, D., F. Bringel, F. H. Schuren, W. M. de Vos, R. J. Siezen, and M. Kleerebezem. 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J. Bacteriol.* 187:6119–27.
- Montrocher, R., M. C. Verner, J. Briolay, C. Gautier, and R. Marmesse. 1998. Phylogenetic analysis of the *Saccharomyces cerevisiae* group based on polymorphisms of rDNA spacer sequences. *Int. J. Syst. Bacteriol.* 48:295–303.
- Moore, K. J., M. G. Johnson, and J. R. Morris. 1988. Indigenous yeast microflora on Arkansas white riesling (*Vitis vinifera*) grapes and in model must systems. *J. Food Sci.* 53:1725–28.
- Mortimer, R., and M. Polsinelli. 1999. On the origins of wine yeast. *Res. Microbiol.* 150:199–204.
- Muyzer, G., and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Ant. van Leeuwenhoek* 73:127–41.
- Neeley, E. T., T. G. Phister, and D. A. Mills. 2005. Differential real-time PCR assay for enumeration of lactic acid bacteria in wine. *Appl. Environ. Microbiol.* 71:8954–7.
- Ness, F., F. Lavallee, D. Dubourdieu, M. Aigle, and L. Dulau. 1993. Identification of yeast strains using the polymerase chain reaction. *J. Sci. Food Agric.* 62:89–94.
- Olsen, G. J., C. R. Woese, and R. Overbeek. 1994. The winds of (evolutionary) change: breathing new life into microbiology. *J. Bacteriol.* 176:1–6.
- Osborne, J. P., and C. G. Edwards. 2005. Bacteria in winemaking. In S. Taylor (ed.), *Advances in Food and Nutrition Research*, 50:139–177.
- Pasteur, L. 1873. *Études sur le vin, ses maladies, causes qui les provoquent, procédés nouveaux pour le conserver et pour le vieillir*, 2. éd., F. Savy, Paris.

- Peynaud, E., and S. Domerco. 1959. A review of microbiological problems in winemaking in France. *Amer. J. Enol. Vitic.* 10:69–77.
- Phister, T. G., and D. A. Mills. 2003. Real-time PCR assay for detection and enumeration of *Dekkera bruxellensis* in wine. *Appl. Environ. Microbiol.* 69:7430–4.
- Pinzani, P., L. Bonciani, M. Pazzagli, C. Orlando, S. Guerrini, and L. Granchi. 2004. Rapid detection of *Oenococcus oeni* in wine by real-time quantitative PCR. *Lett. Appl. Microbiol.* 38:118–24.
- Poblet, M., N. Rozes, J. M. Guillamon, and A. Mas. 2000. Identification of acetic acid bacteria by restriction fragment length polymorphism analysis of a PCR-amplified fragment of the gene coding for 16S rRNA. *Lett. Appl. Microbiol.* 31:63–7.
- Prakitchaiwattana, C. J., G. H. Fleet, and G. M. Heard. 2004. Application and evaluation of denaturing gradient gel electrophoresis to analyze the yeast ecology of wine grapes. *FEMS Yeast Res.* 4:865–77.
- Pramateftaki, P. V., P. Lanaridis, and M. A. Typas. 2000. Molecular identification of wine yeasts at species or strain level: a case study with strains from two vine-growing areas of Greece. *J. Appl. Microbiol.* 89:236–48.
- Pretorius, I. S. 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* 16:675–729.
- Pretorius, I. S., T. J. van der Westhuizen, and O. P. H. Augustyn. 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. a review. *S. African J. Enol. Vitic.* 20:61–74.
- Prust, C., M. Hoffmeister, H. Liesegang, A. Wiezer, W. F. Fricke, A. Ehrenreich, G. Gottschalk, and U. Deppenmeier. 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. *Nature Biotechnol.* 23:195–200.
- Querol, A., E. Barrio, T. Huerta, and D. Ramon. 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. *Appl. Environ. Microbiol.* 58:2948–2953.
- Querol, A., E. Barrio, and D. Ramon. 1994. Population dynamics of natural *Saccharomyces* strains during wine fermentation. *Int. J. Food Microbiol.* 21:315–23.
- Raspor, P., F. Cus, K. P. Jemec, T. Zagorc, N. Cadez, and J. Nemanic. 2002. Yeast population dynamics in spontaneous and inoculated alcoholic fermentations of Zametovka must. *Food Tech. Biotech.* 40:95–102.
- Rawsthorne, H., and T. G. Phister. 2006. A real-time PCR assay for the enumeration and detection of *Zygosaccharomyces bailii* from wine and fruit juices. *Int. J. Food Microbiol.* 112:1–7.
- Renouf, V., M. Falcou, C. Miot-Sertier, M. C. Perello, G. De Revel, and A. Lonvaud-Funel. 2006a. Interactions between *Brettanomyces bruxellensis* and other yeast species during the initial stages of winemaking. *J. Appl. Microbiol.* 100:1208–19.
- Renouf, V., O. Claisse, C. Miot-Sertier, and A. Lonvaud-Funel. 2006b. Lactic acid bacteria evolution during winemaking: use of *rpoB* gene as a target for PCR-DGGE analysis. *Food Microbiol.* 23:136–45.
- Renouf, V., and A. Lonvaud-Funel. 2007. Development of an enrichment medium to detect *Dekkera/Brettanomyces bruxellensis*, a spoilage wine yeast, on the surface of grape berries. *Microbiol Res.* 162:154–167.
- Ribereau-Gayon, J., P. Ribereau-Gayon, and G. Seguin. 1980. *Botrytis cinerea* in enology. In Coley-Smith, J.R., Jarvis, W.R. (ed.), *The Biology of Botrytis*. Academic Press, New York, p. 251–274.
- Ribereau-Gayon, P., D. Dubourdieu, B. Doneche, and A. Lonvaud. 2000. Handbook of Enology Volume 1: The Microbiology of Wine and Vinifications. John Wiley and Sons, LTD, New York, NY.
- Rodas, A. M., S. Ferrer, and I. Pardo. 2003. 16S-ARDRA, a tool for identification of lactic acid bacteria isolated from grape must and wine. *Syst. Appl. Microbiol.* 26:412–22.
- Rodas, A. M., S. Ferrer, and I. Pardo. 2005. Polyphasic study of wine *Lactobacillus* strains: taxonomic implications. *Int. J. Syst. Evol. Microbiol.* 55:197–207.
- Rodrigues, N., G. Goncalves, S. Pereira-da-Silva, M. Malfeito-Ferreira, and V. Loureiro. 2001. Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces*. *J. Appl. Microbiol.* 90:588–99.

- Rodrigues, U. M., and R. G. Kroll. 1985. The direct epifluorescent filter technique (DEFT): increased selectivity, sensitivity and rapidity. *J. Appl. Bacteriol.* 59:493–9.
- Rosini, G., F. Federici, and A. Martini. 1982. Yeast flora of grape berries during ripening. *Microb. Ecol.* 8:83–89.
- Ruiz, A., M. Poblet, A. Mas, and J. M. Guillamon. 2000. Identification of acetic acid bacteria by RFLP of PCR-amplified 16S rDNA and 16S-23S rDNA intergenic spacer. *Int. J. Syst. Evol. Microbiol.* 50:1981–7.
- Sabate, J., J. Cano, A. Querol, and J. M. Guillamon. 1998. Diversity of *Saccharomyces* strains in wine fermentations: analysis for two consecutive years. *Lett. Appl. Microbiol.* 26:452–5.
- Sato, H., F. Yanagida, T. Shinohara, M. Suzuki, K.-i. Suzuki, and K. Yokotsuka. 2001. Intraspecific diversity of *Oenococcus oeni* isolated during red winemaking in Japan. *FEMS Microbiol. Lett.* 202:109–114.
- Schutz, M., and J. Gafner. 1993. Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J. Appl. Bacteriol.* 75:551–558.
- Schutz, M., and J. Gafner. 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* 19:253–257.
- Sipiczki, M. 2003. *Candida zemplinina* sp. nov., an osmotolerant and psychrotolerant yeast that ferments sweet botrytized wines. *Int. J. Syst. Evol. Microbiol.* 53:2079–83.
- Sohier, D., J. Coulon, and A. Lonvaud-Funel. 1999. Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. *Int. J. Syst. Bacteriol.* 49:1075–81.
- Sohier, D., and A. Lonvaud-Funel. 1998. Rapid and sensitive *in situ* hybridization method for detecting and identifying lactic acid bacteria in wine. *Food Microbiol.* 15:391–397.
- Sponholz, W. 1993. Spoilage by wine microorganisms. In Fleet, G. H. (ed.), *Wine Microbiology and Biotechnology*. Harwood Academic Publishers, Switzerland, p. 395–420.
- Stender, H., C. Kurtzman, J. J. Hyldig-Nielsen, D. Sorensen, A. Broome, K. Oliveira, H. Perry-O'Keefe, A. Sage, B. Young, and J. Coull. 2001. Identification of *Dekkera bruxellensis* (*Brettanomyces*) from wine by fluorescence *in situ* hybridization using peptide nucleic acid probes. *Appl. Environ. Microbiol.* 67:938–41.
- Stevenson, K. E., and T. R. Graulich. 1978. Injury and recovery of yeasts and molds. *Adv. Appl. Microbiol.* 23:203–17.
- Stratford, M., P. Morgan, and A. H. Rose. 1987. Sulfur dioxide resistance in *Saccharomyces cerevisiae* and *Saccharomyces ludwigii*. *J. Gen. Microbiol.* 133:2173–2179.
- Tenreiro, R., M. A. Santos, H. Paveia, and G. Vieira. 1994. Inter-strain relationships among wine leuconostocs and their divergence from other *Leuconostoc* species, as revealed by low frequency restriction fragment analysis of genomic DNA. *J. Appl. Bacteriol.* 77:271–80.
- Thornton, R. J. 1991. Wine yeast research in New-Zealand and Australia. *Crit. Rev. Biotechnol.* 11:327–345.
- Torija, M. J., N. Rozes, M. Poblet, J. M. Guillamon, and A. Mas. 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* 80:47–53.
- Torriani, S., G. Zapparoli, and G. Suzzi. 1999. Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. *Ant. van Leeuwenhoek* 75:207–15.
- Towner, K. J., and A. Cockayne. 1993. *Molecular methods for microbial identification and typing*. Chapman & Hall, New York.
- Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solov'yev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* 428:37–43.
- Valente, P., J. P. Ramos, and O. Leoncini. 1999. Sequencing as a tool in yeast molecular taxonomy. *Can. J. Microbiol.* 45:949–58.
- van Vuuren, H. J. J., and C. J. Jacobs. 1992. Killer yeasts in the wine industry - a review. *Amer. J. Enol. Vitic.* 43:119–128.

- Vaughan-Martini, A., and A. Martini. 1995. Facts, myths and legends on the prime industrial microorganism. *J. Ind. Microbiol.* 14:514–22.
- Viti, C., L. Giovannetti, L. Granchi, and S. Ventura. 1996. Species attribution and strain typing of *Oenococcus oeni* (formerly *Leuconostoc oenos*) with restriction endonuclease fingerprints. *Res. Microbiol.* 147:651–660.
- Vitzthum, F., and J. Bernhagen. 2002. SYBR Green I: an ultrasensitive fluorescent dye for double-stranded DNA quantification in solution and other applications. *Recent Res. Devel. Anal. Biochem.* 2:65–93.
- Walling, E., E. Gindreau, and A. Lonvaud-Funel. 2005. A putative glucan synthase gene *dps* detected in exopolysaccharide-producing *Pediococcus damnosus* and *Oenococcus oeni* strains isolated from wine and cider. *Int. J. Food Microbiol.* 98:53–62.
- Ward, A. C. 1992. Rapid analysis of yeast transformants using colony-PCR. *Biotechniques* 13:350.
- Wilson, K. H., W. J. Wilson, J. L. Radosevich, T. Z. DeSantis, V. S. Viswanathan, T. A. Kuczmarski, and G. L. Andersen. 2002. High-density microarray of small-subunit ribosomal DNA probes. *Appl. Environ. Microbiol.* 68:2535–2541.
- Winzler, E. A., C. I. Castillo-Davis, G. Oshiro, D. Liang, D. R. Richards, Y. Zhou, and D. L. Hartl. 2003. Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. *Genetics* 163:79–89.
- Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, C. Fritze, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, V. Lelaure, et al. 2002. The genome sequence of *Schizosaccharomyces pombe*. *Nature* 415:871–880.
- Xu, H., N. Roberts, F. Singleton, R. Attwell, D. Grimes, and R. Colwell. 1982. Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. *Microbial Ecol.* 8:313–323.
- Xufre, A., H. Albercaria, J. Inacio, I. Spencer-Martins, and F. Girio. 2006. Application of fluorescence *in situ* hybridization (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int. J. Food Microbiol.* 108:376–84.
- Xufre, A., F. Simoes, F. Girio, A. Clemente, and M. T. Amaral-Collaco. 2000. Use of RAPD analysis for differentiation among six enological *Saccharomyces* spp. strains. *Food Tech. Biotechnol.* 38:53–58.
- Zapparoli, G., S. Torriani, P. Pesente, and F. Dellaglio. 1998. Design and evaluation of malolactic enzyme gene targeted primers for rapid identification and detection of *Oenococcus oeni* in wine. *Lett. Appl. Microbiol.* 27:243–6.
- Zavaleta, A. I., A. J. Martinez Murcia, and F. Rodriguez Valera. 1997. Intraspecific genetic diversity of *Oenococcus oeni* as derived from DNA fingerprinting and sequence analyses. *Appl. Environ. Microbiol.* 63:1261–1267.
- Ze-Ze, L., R. Tenreiro, and H. Paveia. 2000. The *Oenococcus oeni* genome: physical and genetic mapping of strain GM and comparison with the genome of a 'divergent' strain, PSU-1. *Microbiology* 146:3195–204.
- Zhou, J. 2003. Microarrays for bacterial detection and microbial community analysis. *Curr. Opin. Microbiol.* 6:288–94.

Chapter 7

Beer Production

Giuseppe Comi and Marisa Manzano

Abstract Different methods are used to follow the microbial populations during beer fermentation and maturation. In recent years biomolecular methods, based on the use of PCR, have been developed to study the fate of implicated microorganisms in beer fermentation. These methods allow for the evaluation of yeasts during the fermentation phase, and the bacteria or yeasts responsible for the beer alteration. Brewing yeasts characterization and contaminant bacteria identification using fast molecular methods such as PCR, RAPD-PCR, PCR-TTGE, PCR-DGGE, short tandem repeats (STR) analyses are now important tools to improve beer quality. This chapter explains the main biomolecular methods developed and optimized to identify brewing yeasts and bacteria by culture-dependent and culture-independent methods.

1 Introduction

The manufacture of beer is a biological process whereby barley and hops, both agricultural products, are converted by a complex biochemical process into beer by controlling biochemical reactions in the malting, mashing and fermentation stages. Despite recent changes in the brewing industry, many brewers follow traditional production methods, using technology that has not changed in 200 years. These brewers are afraid that change might negatively affect the quality or image of their beer. Fortunately new industry and mergers to form big brewing groups sustained by new technological breakthroughs are increasing in many parts of the world. Technological innovations have been applied to the brewing process to increase productivity and quality, to save energy or to create new products (Iserentant 1994). However despite the use of new plants and new additives, the brewing process and the problems created by microorganisms have remained the same. Innovation has reduced some microbial problems, but it has not eliminated them completely. The beer process includes: the choice of raw materials and water, hops, and yeasts; the wort production (filtration, boiling); the wort fermentation and maturation. These process steps are briefly described below.

1.1 The Choice of Raw Materials

Choosing the malt is the first step of the brewing process. The malt contains starch, which is saccharified by the endogenous enzymatic activity of the grain. Malted barley is the normal starch source. The choice of big or small grain depends on the brewer, with most preferring two-row spring barley with big grains. Sometimes brewers may add other starch sources (rice, corn, wheat) to the wort, or even sugar syrups to increase the sugar concentrations (Kendall 1995).

The malting process includes the germination of the grain, followed by a heat treatment, which stops the germination. The barley grains are steeped in water at 10 to 15 °C for two to three days to achieve a grain moisture content of approximately 45 percent to 50 percent (w/w), and then are left for four to seven days to germinate. Sprouts or germs are removed, leaving the malt, which is then dried to remove most of the moisture – approximately 4 percent to 6 percent by the end of the process.

The following heat treatment can be modified to obtain different types of malt (caramel, chocolate, Munich) that can be used to produce traditional or special beers.

The brewer then chooses the water, which is very important for the beer quality, and yeast activity, representing up to 90 percent of the finished beer. The water must be a mineral type because some salts and ions (Mg, Zn) are determinant for a rapid and regular fermentation. Salt and ion concentration, in association with other factors, define the typical character of some special beers.

The aroma of beer and, in particular, the bitter aroma comes from the hops which have been used in brewing since the Middle Ages. Hops are added to the wort during the boiling process to produce the isomerization of the α -acids. Some brewers also add fresh hops (dry hopping) to the beer during fermentation to increase the volatile acids, responsible for the aroma, which may have been lost during boiling. Several companies sell hop extracts that are pre-isomerized under aqueous conditions and the modified isomerized hop extracts have a higher bittering potential and improve the shelf-life stability of the beer, limiting the deterioration of flavor (called sun-struck flavor) due to sunlight (Iserentant 1994).

The choice of yeasts is an important phase of beer production, as they possess a number of important characteristics that affect the flavor of the beer. Rapid and relevant carbohydrate fermentation ability, appropriate flocculation and sedimentation characteristics, genetic stability, osmotolerance (e.g., the ability to ferment concentrated carbohydrate solutions in high gravity beer), ethanol tolerance and the ability to produce esters, higher alcohols and flavors, high cells viability for repeated recycling and temperature tolerance are the main characteristics evaluated. Brewer's yeasts include only the genus *Saccharomyces* (Hammond 1996; Boulton and Quain 2001). The strains are usually polyploid or aneuploid and do not sporulate. Lager and ale are the two main types of beer produced by a large number of strains belonging to the *Saccharomyces sensu stricto* complex. In particular, strains used in beer production belong to the species *S. cerevisiae* and *S. pastorianus* (synonym *S. carlsbergensis*, a strain obtained from a natural interspecific fusion-cross

between *S. cerevisiae* and *S. bayanus*). The brewers use different strains, or species of physiological race according to the fermentation conditions. A distinction is made between top-fermenting yeasts (mainly belonging to *S. cerevisiae*) and bottom-fermenting yeasts (mainly belonging to *S. carlsbergensis* and *S. uvarum*) (Iserentant 1994).

Top-fermenting yeasts are used to produce ales that are fermented at high temperatures (18 to 25 °C). In this production the yeast biomass floats at the top of the fermented wort until the end of the fermentation. These ale yeasts are less flocculent than lager yeasts; the cells are carried to the fermenting wort surface absorbed into carbon dioxide bubbles.

The opposite occurs when making lagers, which are fermented at lower temperatures (8 to 12 °C), using bottom-fermenting yeasts. The bottom yeasts differ from the top-fermenting ones in their capability to use raffinose and melibiose and flocculate to the bottom of the tank at the end of fermentation. If both lager and ale beers are centrifuged after fermentation to separate the yeasts, it becomes possible to use non-flocculent yeast strains. Usually brewer's yeast strains are incapable of fermenting all the sugars of the wort. They are able to ferment the sucrose, fructose, glucose, maltose and maltotriose, but not dextrin material of the wort. The transport, hydrolysis and fermentation of maltose are particularly important in brewing, distilling and baker's yeast strains since maltose is the major sugar component of brewing wort, spirit mash and wheat dough.

1.2 Wort Production

Malt is milled to produce better transformations and to increase the solution of extractable material. Sometimes the grains are steam conditioned to improve moisture content. Then the malt flour, mixed with water, is heated to permit the enzymatic degradation of the substrate. The high-molecular-weight compounds are hydrolyzed in short molecules that yeasts can use during the fermentation. In fact the mashing process is a series of enzymatic reactions whereby most of the insoluble unfermentable starch and proteins are hydrolyzed to soluble fermentable materials (Briggs, et al. 2004).

The wort is subjected to filtration by a filter press to separate the spent grain from the liquid, and collected in a wort boiling kettle and boiled with the hops for 60 to 90 minutes to inactivate the amylases, the proteases and the glucanases. In addition the temperatures result in sterilization useful in any subsequent fermentation. After boiling for one or two hours the proteins coagulate and precipitate and this plays an important role in beer stabilization. Flavors expand by increasing the isomerized acids and products of Maillard reactions. Complex chemical changes occur in the compounds extracted from the hops by boiling. The typical hops compounds, humulone and lupulone, may be converted to soft resins by oxidation and polymerization. Both acids possess antiseptic properties and, together with essential oils in hops, produce the characteristic flavors and aromas of beer. The tannin

of hops is converted to phlobaphene which complexes with proteins during boiling to form a precipitate. In addition off-flavors such as sulphide compound evaporate. Finally, during the boiling the wort becomes concentrated by about 8 percent to 10 percent.

1.3 Wort Fermentation and Maturation

Before adding the yeast, the trub of the spent hops and all the coagulated compounds (hot break) are removed by filtration, centrifugation or sedimentation by a whirlpool, a centrifuge or a hot setting tank. Then the wort is cooled, oxygenated and inoculated with a pure culture of yeast starter. The yeast strains, the temperature and the duration of the fermentation depend on the type of beer. Maltose and smaller amounts of fructose, glucose, maltotriose and sucrose are converted into alcohol and into the most important flavors during fermentation. The main flavors include esters (ethylacetate, isoamylacetate) and higher alcohols (butanol, isoamylalcohol, propanol). They all determine the character of the final beer. At the end of fermentation the yeasts flocculate in the cylindroconical vessels and can be harvested for future production (Kunze 1999; Briggs, et al. 2004).

After removing the flocculated yeasts the beer is matured. The green beer is allowed to age or mature under cool conditions (at 2°C) for periods varying between two weeks and two months, depending on the type of beer. In this phase the remaining sugar can be fermented and transformed into alcohol and CO₂, which saturate the beer. Diacetyl, a by-product of isoleucine/valine synthesis formed by the decarboxylation of α -acetolactate, is reduced by yeasts into acetoin and butanediol, both flavor-neutral compounds. The classical maturation lasts several weeks and ends with a complete yeast flocculation, which brings a total clarification of the beer. The beer can be filtered, bottled and ready for consumption.

2 Biomolecular Methods

Traditional laboratory methods do not always provide the necessary specificity and sensitivity to cultivate and identify in real-time beer microorganisms. The use of selective media and the incubation conditions still appear to be the method preferred by breweries, but they are time consuming. The media used depend strictly on the type of sample and on the specificity and sensitivity required. In addition, in order to detect all members within a group of specific beer microorganisms, different media must be used. Considering the requests of brewers to utilize fast, sensitive and specific methods to know in real-time the behavior of microorganisms during beer production, the recent aim of many researchers has been to develop and optimize methods based on Polymerase Chain Reaction (PCR) and biomolecular techniques. In the last few years applied molecular microbiology has been a fast

moving area. One of the branches of this discipline is involved in the development of molecular methods for the identification and monitoring of microorganisms in natural ecosystems (Ercolini 2004). The biomolecular methods in modern microbiology have become a valid support to traditional techniques. The study of a bacterial community and its evolution in environmental samples needs molecular identification methods, especially those including the sequencing of gene coding for ribosomal 16S rRNA (Ercolini 2004). Many biomolecular methods, based on culture-independent techniques, are developed and performed to characterize the microorganisms that interact during beer fermentation. Today many researchers consider molecular methods to be characterized by rapidity and reliability, and for this reason their main aim is to produce methods to use in real-time and to ascertain the quality in each phase of beer production.

The biomolecular methods are employed for both identification and typing yeasts responsible for the beer production and for identification of microorganisms, mainly yeasts and bacteria, responsible of beer spoilage.

2.1 Characterization of Brewing Yeasts by Molecular Methods

The PCR-based methods used so far in beer fermentations are: PCR-Restriction Fragment Length Polymorphism (RFLP) analysis, Temporal Temperature Gel Electrophoresis (TTGE)-PCR, Denaturing Gradient Gel Electrophoresis (DGGE)-PCR and microsatellites analysis.

Rainieri, et al. (2006) evaluated the genetic variability of two lager beer species, *S. bayanus* and *S. pastorianus*, by using PCR-RFLP analysis of 48 genes and partial sequences of 16. Within these two species they identified “pure” strains containing a single type of genome and “hybrid” strains that contained portions of the genomes from the “pure” lines, as well as alleles termed “lager” that represent a third genome commonly associated with lager brewing strains. *S. uvarum* and *S. bayanus* represent the two pure lines, while the hybrid lines identified include *S. cerevisiae/S. bayanus*/Lager, *S. bayanus/S. uvarum*/Lager and *S. cerevisiae/S. bayanus/S. uvarum*/Lager. They suggest that the genome of the lager strains could have resulted from chromosomal loss, replacement or rearrangement within the hybrid genetic lines.

Manzano, et al. (2005) used classical microbiological methods in association with molecular methods (DNA amplification, TTGE and DGGE) to rapidly analyze microbial communities on the basis of sequence-specific separation of DNA amplicons. The primers used for the yeast strain analysis, named Schaf (forward primer) (5'- GTAGTGAGTGATACTCTT-3') and Schar (reverse primer) (5'- AGAACACATGTTGCCTAGAC-3'), targeting the Internal Transcribed spacers (ITS), were specific for *Saccharomyces sensu stricto* and amplified a 207bp fragment. In this study bacterial populations were also analyzed by the use of primers P1 (5'-CGCGCGTGCCTAATACATGC-3') and primer P2 (5'-TTCCCACGGCTTACTCACC-3'), targeting the V1 region of the 16S rRNA,

according to the protocol proposed by Cocolin, et al. (2001). TTGE allowed the comparison of the different *Saccharomyces cerevisiae* strains used in brewing (Fig. 7.1), whereas DGGE allowed the identification of lactic acid bacteria (LAB) in beer. These methods proved to be reliable tools for fast comparison of strains of *S. cerevisiae* collected from different craft breweries where they were used as starters, to check the presence of possible yeast contaminants in the brewing process and for rapid LAB identification.

On the basis of the of the point mutation present in the amplicon sequences, it was possible to differentiate yeast starters used in beer production and to detect wild *S. cerevisiae* strains not belonging to any of the starters used. The results were available in a short time; in fact, the identifications were available within eight hours. PCR-DGGE could be used for the identification of contaminants or in strain differentiation without the need of complicated biochemical tests. The 44 LAB isolated from MRS agar were identified by the DGGE method as *Kokuria* sp., *Lactobacillus brevis*, *Lb. sakei*, *Lb. lactis*, *Lb. hilgardii* and *Pediococcus parvulus*, and these results were in accordance with the data of Satokari, et al. (1998) and Juvonen and Satokari (1999).

Giusto, et al. (2005, 2006) used PCR-TTGE, RAPD (Random Amplified Polymorphic DNA) -PCR and Restriction Enzyme Analysis (REA) techniques to analyze yeasts isolated from craft beers produced in Northeast Italy. Usually micro-breweries use yeasts supplied by Italian or foreign industrial breweries for beer production. Yeast species are often unknown; moreover the vitality, the viability, the physiological state and the number of generation are not known. To improve the consistency and quality of fermentations it is important to evaluate the physiological

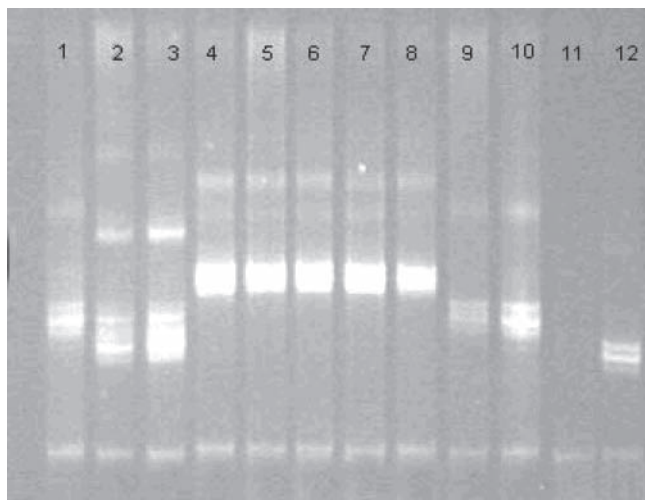


Fig. 7.1 TTGE migration patterns of several strains of *S. cerevisiae* isolated from beer. Lanes 1 to 10, isolates; lane 11, negative control; lane 12, *S. cerevisiae* ATCC 36024 (Manzano, et al. 2005, reproduced with permission)

state of the yeast strain used (Mochaba, et al. 1997) and the LAB contamination. The methods proposed by Giusto, et al. (2005, 2006) allowed a fast identification of the strains analyzed as *S. cerevisiae* (within eight hours), and a fast intraspecific differentiation of the 28 yeast strains tested (within 18 hours) (Fig. 7.2). The M13 primer used in the RAPD-PCR technique by Giusto, et al. (2006) allowed intraspecific differentiation within eight hours after cell growth on the isolation media used (Fig. 7.3).

Universally primed PCR analysis, microsatellite fingerprinting and PCR-RFLP of the ribosomal ITS were used by Naumova, et al. (2002) to identify genetic relationships of 24 phenotypically different strains isolated from sorghum beer in West

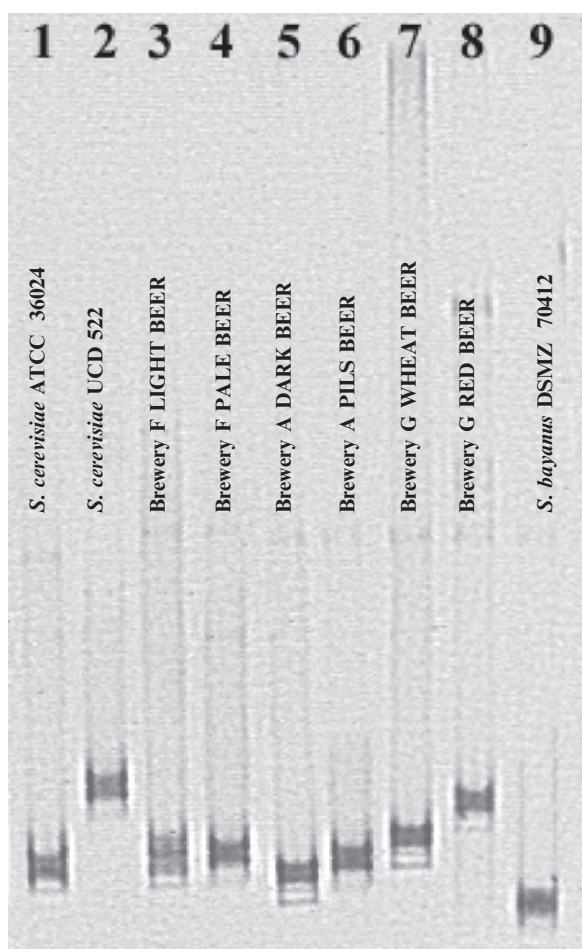


Fig. 7.2 TTGE analysis of *S. cerevisiae* strains isolated from craft beers. Lane 1, *S. cerevisiae* ATCC 36024; lane 2, *S. cerevisiae* UCD 522; lane 3, light beer strain; lane 4, pale beer strain; 5, dark beer strain; lane 6, pils beer strain; lane 7, wheat beer strain; lane 8, red beer strain; lane 9, *S. bayanus* DSMZ 70412 (Giusto, et al. 2006, reproduced with permission)

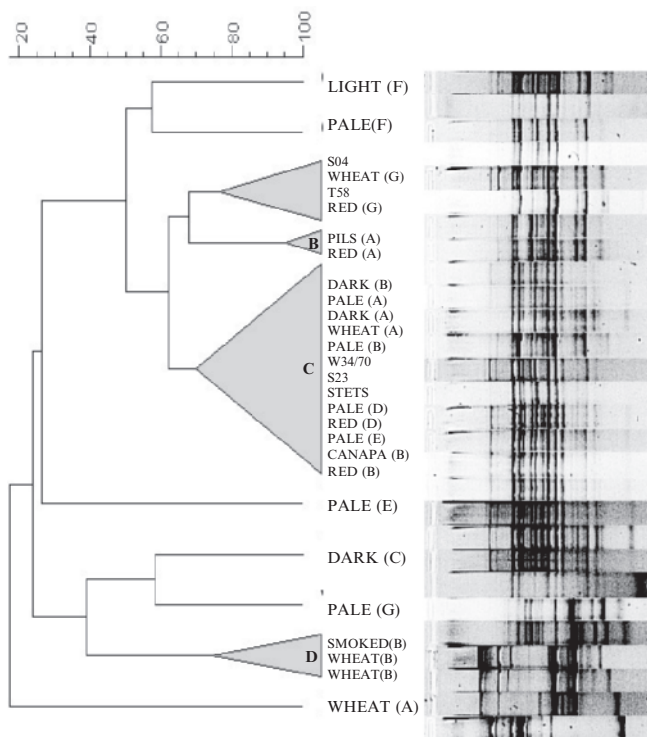


Fig. 7.3 UPGMA-based dendrogram obtained after elaboration of RAPD-PCR profiles. The coefficient of correlation of 70 percent was selected to differentiate the clusters. Strains analyzed: LIGHT (F), light beer strain; PALE (F), pale beer strain; S04, dry yeast; WHEAT (G), wheat strain; T58, dry yeast; RED (A), red beer strain; PILS (A), Pils beer strain; RED (A), red beer strain; DARK (B), dark beer strain; PALE (A), pale beer strain; DARK (A), dark beer strain; WHEAT (A), wheat beer strain; PALE (B), pale beer strain; W34/70, dry yeast; S23, dry yeast; STE TS, slurry yeast; PALE (D), pale beer strain (Giusto, et al. 2006, reproduced with permission)

Africa and the type cultures of the *Saccharomyces sensu strictu* species. The authors demonstrated that ITS-PCR-RFLP analysis with the endonucleases *Hae*III, *Hpa*II, *Scr*FI and *Taq*I was useful for discriminating *S. cerevisiae*, *S. kudriavzevii*, *S. mikalae* from each other and from the *S. bayanus*/*S. pastorianus* and *S. cariocanus*/*S. paradoxus* pairs. The type culture of *S. cerevisiae* CBS 1171 exhibited the same restriction patterns as the sorghum beer strains. The PCR profiles generated with the microsatellite primer (GTG)₅ and with the universal primer N21 of the control strain CBS 1171 were almost identical to all isolates. Naumova, et al. (2002) concluded that, despite phenotypic peculiarities, the strains involved in sorghum beer production in Ghana and Burkina Faso belonged to *S. cerevisiae*, even though the sequencing of the rDNA ITS1 region and Southern hybridization analysis demonstrated that these strains represented a divergent population of *S. cerevisiae*.

Tornai-Lehoczki and Dlačny (2000) investigated different methods to group ale and lager strains, including the electrophoretic karyotyping, RAPD analysis and the

RFLP of 18S rRNA-coding DNA. They found the same RFLP pattern was present in all production brewer yeast strains (type strain and synonym type strains of *S. cerevisiae*), and demonstrated only a small difference existing between the type and synonym type strains of *S. pastorianus*. For this reason all production brewing yeast strains they investigated seemed to belong to *S. cerevisiae*. They concluded that electrophoretic karyotyping and RAPD analysis appeared to be suitable methods for distinguishing not only the type and synonym type strain of *S. cerevisiae* and *S. pastorianus*, but also the ale and the lager strains.

To differentiate brewing from non-brewing yeasts a specific PCR, which targeted the open reading frame of *FLO1*, was employed by Yamagishi, et al. (1999). The *FLO1* gene allows the flocculation and is spread among yeasts in different polymorphisms so it can be used to distinguish between non-brewing yeasts, including non-brewing *Saccharomyces* yeasts, non-*Saccharomyces* yeasts and brewing yeasts. The primers for the *FLO1* gene were FL1 (5'-CCA AAA TGA CAA TGC CTC GCT AT-3') and FLR2 (5'-CCA TTG CTA GGA TAG AAT GGG GTA ATA ATT GGA CG-3'). The results demonstrated that the molecular sizes of the PCR products differed between brewing and non-brewing *Saccharomyces* yeasts, and no *FLO1* PCR products were obtained from non-*Saccharomyces* yeasts. To complete the differentiation of the strains an additional method, based on specific RFLP-PCR, was used. The specific primers amplified the region between the 5S and 26S rRNA genes and the amplicons were digested with restriction enzymes *ScrfI* and *MpsI*. Different restriction profiles were obtained from brewing and non-brewing yeasts.

The development of techniques to analyze brewing yeast strains includes the use of non-radioactive probes. The use of DNA fingerprinting to analyze brewer's yeast and distinguish brewer from non-brewer strains was born many years ago. Wightman, et al. (1996) analyzed the brewing strains *S. cerevisiae* by DNA fingerprinting, using a Southern blotting and hybridization procedure and employing the Tyl-15 transposon as a probe. The success of these methods is dependent on the restriction enzyme used to digest the DNA prior to Southern blotting and hybridization. The authors found that *EcoRI*, *PstI* and *SalI* were particularly useful in readily differentiating between strains. The method they proposed permitted the differentiation of both ale and lager yeasts, and was sufficiently sensitive to distinguish between very closely related strains. DNA fingerprinting by this approach confirmed that a flocculent strain isolated during a production-scale fermentation with a lager yeast was genotypically different from the parent.

Delta (δ) sequences were used to discriminate brewery ale and lager yeast strains. By using two different PCR approaches, Coakley, et al. (1996) obtained a rapid discrimination of closely related *S. cerevisiae* strains, a particularly challenging task for breweries that are using, closely related strains simultaneously to manufacture different products. The authors solved the problem by using sets of primers targeting the δ sequences in PCR and RAPD-PCR. For the amplification of yeast genomic DNA situated between δ sequences, primers $\delta 1$ (5' CAAATTCTCACCTATA/TTCTCA-3') and $\delta 2$ (5'-GTGGTTTTTATTCCAACA-3') were used. For RAPD

analysis a total of nine primers were employed, but in the study it was proposed that the primer 539 (5'-TAAAATAAGGAGATTATTATG-3') target the δ sequence. The use of δ sequence as a target could rapidly differentiate between many ale and lager strains, so the authors obtained characteristic profiles of both lager and ale yeasts. They suggested the method for differentiation of yeasts isolated from brewery wort or from active dry yeast preparations as being sensitive, specific, and fast. Other non-traditional techniques have been developed to study yeast strains during beer production. In recent years the proteomic technologies represent the new way to understand the dynamics of yeasts during beer production.

Kobi, et al. (2004) presented the first protein map of an ale-fermenting yeast. They identified 20 spots corresponding to 133 different proteins. By comparing the proteome of the ale strain with a lager brewing yeast and the *S. cerevisiae* strain S288c (Yeast genetics Stock Center, University of California, Berkeley, CA, US) they confirmed that the ale strain is much closer to S288c than to the lager strain at the proteome level. They followed the dynamics of the ale-brewing yeast proteome during production-scale fermentation from the beginning to the end of the first and the third usage of the yeast. The proteomic studies discovered that most of the changes in yeasts during the first generation were due to the switch from aerobic propagation to anaerobic fermentation. Vice versa fewer changes were observed during the third generation, even though the subsequent generations produced stress-response proteins. It was concluded that the ale brewing yeast strain appears to be well adapted to fermentation conditions and stress.

The study, which provides the first example of using proteome analysis for investigating taxonomic relationships between divergent yeast species, was made by Joubert, et al. (2000). They used two-dimensional (2-D) gel electrophoresis to analyze the proteomes of different *Saccharomyces* species isolated from breweries. The aim was to obtain information on the identity of the parental strains that gave rise to industrial lager yeasts. It should be considered that modern lager brewing yeasts used in beer production are hybrid strains consisting of at least two different genomes. It was found that the proteome of lager brewing yeasts and of type strains of *S. carlsbergensis*, *S. monacensis* and *S. pastorianus* can be interpreted as two elementary patterns. The first originated from *S. cerevisiae*-like proteins and the second from a divergent *Saccharomyces* species, a particular *S. pastorianus* strain NRRL Y-1551. The studies produced a 2-D map of industrial lager brewing yeasts by comparing their protein spots to known *S. cerevisiae* proteins. This map can be accessed on the Lager Brewing Yeast Protein Map server through the World Wide Web (<http://www.ibqc.u-bordeaux2.fr/YPM/>).

At last the transcriptome of a lager brewing yeast (*Saccharomyces carlsbergensis*, syn. of *S. pastorianus*) was analyzed at 12 different time points spanning a production-scale lager beer fermentation by Olesen, et al. (2002). Generally, the increased RNA expression was observed at the beginning of the fermentation and the transcribed genes included protein and lipid biosynthesis or glycolysis.

2.2 Detection and Characterization of Spoiling Yeasts and Bacteria in Beer

During yeast fermentation and beer maturation many bacteria can grow and produce turbidity, off-flavors and off-odors. The Gram positive bacteria are generally considered to be the most problematic organisms in modern breweries. The common spoilers of finished (packaged) beer are different strains of lactobacilli, pediococci, micrococci and of strictly anaerobic bacteria. *Lb. brevis*, *Lb. buchneri*, *Lb. coryneformis*, *Lb. lindneri*, *Lb. curvatus*, *Lb. casei*, *Lb. plantarum*, *Lb. brevisimilis*, *Lb. malefermentans*, *Lb. parabuchneri*, *P. damnosus*, *P. inopinatus*, *P. dextrinicus* and *Micrococcus kristinae* are often relevant to brewing. Strictly anaerobic Gram negative bacteria, including *Pectinatus cerevisiiphilus*, *Pectinatus frisingensis*, *Selenomonas lacticifex*, *Zymomonas raffinivorans* and *Megasphaera cerevisiae* (Chelack and Ingledew 1987; Jespersen and Jakobsen 1996; Vaughan, et al. 2005), a spoilage microorganism of low alcohol beer, are apparently increasing in importance. *Zymomonas mobilis*, an anaerobic but oxygen-tolerant microorganism, is capable of spoiling primed beer (Jespersen and Jakobsen 1996). Wort can also be spoiled by coliforms, although they are considered occasional beer spoilers. The improved beer technology process has decreased the importance of Gram negative aerobic bacteria, which could survive during main and secondary yeast fermentation as beer spoilers (Jespersen and Jakobsen 1996).

In the recent years additional reports became available to identify yeasts and LAB, and to follow their fate in beer, considering they may be detrimental to the quality of many fermented beers. LAB spoilage occurs either during the main or the secondary fermentation stages or during the storage of beer. Many cocci, pediococci and some strains of *Leuconostoc mesenteroides* can produce exopolysaccharidic compounds that modify beer viscosity and lead to ropiness. They can produce acetic acid, or diacetyl, that influences the beer's aroma. Wild yeasts and LAB are recognized for their ability to improve beer quality, but are also producers of off-flavors and off-odors. For these reasons many biomolecular studies on beer microorganisms evaluate the fate of spoiling bacteria and yeasts. Until now the few data about the problem of contamination of "wild yeasts," which usually appears after the third usage during the production-scale fermentation, are due to the fact that yeasts are added to wort as starter in high concentration and they always predominate on the other microorganisms.

Since the phenotypic tests and DNA-DNA probes are not sufficiently accurate and the use of 16S RNA genes produces intraspecies heterogeneity, new biomolecular methods are being investigated to learn which microorganisms in real-time interact in beer. Different methods based on DNA-fingerprinting and PCR are proposed.

A taxonomic study about different isolates of *Saccharomyces* spp. identified as contaminants ("wild yeast") in 24 different lager breweries was conducted by Jespersen, et al. (2000). Different methods were performed, including phenotyping, Chromosome Length Polymorphism (CLP), RFLP-PCR and detection by a probe

of *MAL* loci. The yeasts belonged to the *Saccharomyces sensu strictu* complex: 58 percent of the isolates were identified as *S. cerevisiae*, 26 percent as *S. pastorianus* and 3 percent as *S. bayanus*. Different strains (13 percent) could not be identified to the species level based on their phenotypic characteristics, although some of these isolates were identified as *S. cerevisiae* by *Hae*III restriction digest of PCR-ITS regions. By CLP the majority of the brewing contaminants could be grouped as either *S. cerevisiae* or *S. pastorianus*/*S. bayanus*. CLP differentiated between almost all brewing contaminants and separated them from any specific lager brewing yeast. A greater discrimination was obtained by the study of *MAL* loci; the high number of *MAL* loci found in the *Saccharomyces* brewing contaminants indicate their adaptation to a maltose-enriched environment.

Tsuchiya, et al. (1992) developed a sensitive detection and identification PCR method for LAB beer spoilage microorganisms. The method allowed the detection and identification of a 117bp product from the target sequence of the 5S rRNA region, specific for *Lb. brevis*. It was possible to detect one cell when a pure culture of *Lb. brevis* was used, whereas the detection limit was about 30 cells when a mixed culture was used. In the same study, a set of primers to detect a PCR product of 100bp for the 5S rRNA gene of *S. cerevisiae* was designed. When the primers for *Lb. brevis* and *S. cerevisiae* were used in a mixture, both microorganisms were quickly and clearly distinguished.

Stewart and Dowhanick (1996) examined different sets of primers to obtain PCR products from DNA targets of some *Lactobacillus*, *Leuconostoc* and *Pediococcus* strains present during beer fermentation and maturation. The developed primers did not amplify yeasts or non-LAB. However, a yeast cell concentration higher than 3×10^4 could inhibit the LAB detection. The yeast interference was overcome by a nested PCR protocol which utilized a different set of primers in the second step. In fact, the detection of a low bacteria level was also possible in the presence of 10^8 yeast cells, a number commonly present during beer fermentation. The performed method was fast, specific and sensitive and the authors concluded that the procedure assesses bacterial contamination in a few hours and before the end of fermentation, and is the decision point for collecting yeast for re-pitching.

Tompkins, et al. (1996) used commercially available RAPD primers to identify and characterize beer-contaminating bacteria. They used four different primers in the RAPD-PCR to characterize the species of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and other bacteria (*Lb. brevis*, *P. damnosus*, *Pectinatus cerevisiiphilus*, *Lc. mesenteroides*, *Lb. plantarum*, *Meghasphaera cerevisiae*, *Streptococcus raffi-nolactis*) responsible for beer spoilage. The four primers were OPA-04 (5'-AATCGGGCTG), OPA-17 (5'-GACCGCTTGT), OPB-16 (5'-TTTGCCCGGA) and OPE-05 (5'-TCAGGGAGGT) coming from Operon Technologies (Alameda, CA). Moreover, each primer produced a unique and characteristic core fingerprint pattern and allowed differentiation of *S. cerevisiae* var. *carlsbergensis* from *S. cerevisiae* var. *cerevisiae* and *S. cerevisiae* var. *diastaticus*. The use of the core patterns enabled known and unknown contaminants to be distinguished and recognized.

Funahashi, et al. (1998) detected and identified two novel species of *Lactobacillus* as beer-spoilage in the brewing environment. One species was obligate

heterofermentative *Lactobacillus* sp. which had a strong beer-spoilage ability, whereas the second species was a facultative heterofermentative *Lactobacillus* sp. with a weak beer-spoilage ability. Both microorganisms were considered new species in the study of their carbohydrate utilization pattern, ribotype, and DNA-DNA hybridization analysis. However, the obligate heterofermentative *Lactobacillus* strains revealed in the 16S ribosomal DNA sequence a 99.5 percent similarity to *Lb. collinoides*, indicating that these species are related.

Satokari, et al. (1998) detected strictly anaerobic bacteria of the genera *Megasphaera* and *Pectinatus* – which cause off-flavors, off-odors, and turbidity in beer – using a PCR method, since the detection of these microorganisms is often impossible and complicated by traditional methods, and requires long periods of incubation. To improve the results they applied a colorimetric microplate hybridization assay after PCR. A new biotinylated primer was used to amplify a 403 base pair (bp) fragment of the *Megasphaera cerevisiae* 16S rRNA gene. For the amplification of an 816bp fragment of the *Pectinatus* 16S rRNA, two primers from literature were used. Both biotinylated PCR products were captured by streptavidin and hybridized with a digoxigenin-labeled oligonucleotide probe. In the final step of the microplate hybridization method, an enzyme-linked antibody and a colorimetric reaction were used. This method allowed detection of 5×10^3 CFU *Megasphaera cerevisiae*/100 ml beer and 5×10^5 CFU *Pectinatus frisingensis*/100 ml beer.

Juvonen and Satokari (1999) performed a PCR assay for quickly detecting and identifying different strains of *Lb. lindneri*, *Lb. brevis*, *Megasphaera cerevisiae* and *Pectinatus* spp. The assay consisted of an easy sample treatment and specific primers. Artificially contaminated beer samples (obtained by the addition of mixtures of different dilutions of spoilage bacteria) were mixed with a pre-enrichment broth to support the growth of lactobacilli and anaerobic beer spoilers. In this way it was possible to detect a low level of lactobacilli (< 10 CFU/100 ml) after one to three days; a low level of *Pectinatus* spp. after two to four days, and a low level of *Megasphaera cerevisiae* after two to three days of pre-enrichment, depending on the strain and the alcohol content of beer. The assay described, which allowed species- or genus-level detection of the most harmful beer spoilage bacteria in finished beer, was sensitive and time-saving compared to corresponding conventional methods, and simple enough for routine work.

Suzuki, et al. (2004) examined the *horA* homologues and adjacent DNA regions identified in beer-spoiler *Lb. lindneri* and *Lb. paracollinoides* and compared with the corresponding DNA region of beer-spoiler *Lb. brevis*, a strain in which the hop-resistance gene *horA* was originally identified. They selected the region ORFB1-B5, surrounding the *horA* gene and conserved in all the strains they investigated, as the PCR target. They designed specific primers to the adjacent ORFs and could differentiate PCR beer spoilage associated *Lactobacillus* strains from non-spoilers. Applying this PCR method to 92 lactobacilli strains Suzuki, et al. (2004) suggested that the ORF1-B5 region has been acquired by beer spoilage lactobacilli through horizontal gene transfer and provides a theoretical basis for applying a trans-specific genetic marker such as *horA* to deal with unencountered species of beer-spoilage lactobacilli. This hypothesis also provides a theoretical basis for applying trans-species genetic markers to the quality control procedures of breweries.

The microbial composition of biofilms from a beer bottling plant was also studied by a cultivation independent analysis of the 16S rRNA genes by Timke, et al. 2005. Clone libraries were constructed from amplified the 16S rRNA gene and, after differentiation by restriction analysis, representative clones were sequenced. The diversity of the clone libraries was comparable with the diversity found for environmental samples. By this analysis the genus *Methylobacterium* appeared to be one of the dominating groups of the clone libraries. The size of this population was assessed by fluorescence *in situ* hybridization and fatty acid analysis. The multi-methods used assigned uncultivated organisms a considerable number of clones.

3 Concluding Remarks

The novel molecular approaches to monitor microorganisms in food are not commonly used in beer as in other food fermentations. Studies will be needed to assess and possibly improve their effectiveness for the identification and typing of yeasts involved in the fermentation, as well as undesirable spoiling agents involved in the manufacture and storage of beer.

References

- Boulton, C., Quain, D., 2001. Brewing Yeast. In: Brewing yeast and fermentation. Blackwell Science Company, Ames, pp. 143–259
- Briggs, D.E., Boulton, C.A., Brookes, P.A., Stevens, R., 2004. Wort boiling, clarification, cooling and aeration. Metabolism of wort by yeast. In: Brewing Science and practice. TJ International, Cornwall, 322–378, 417–484
- Chelack, B.J., Ingledew, W.M., 1987. Anaerobic Gram Negative Bacteria in Brewing. A Review. J. Am. Soc. Brew. Chem. 45, 123–127.
- Coakley, M., Ross, R.P., Donnelly, D., 1996. Application of the Polymerase Chain Reaction to the rapid analysis of brewery yeast strains. J. Inst. Brew. 102, 349–354.
- Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing Gradient Gel Electrophoresis Analysis of the 16S rRNA gene V1 region to Monitor the Dynamic Changes in the Microbial Population during the fermentation of Italian Sausages. Appl. Environ. Microbiol. 67, 5113–5121.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection. J. Microbiol. Methods 56, 297–314
- Funahashi, W., Suzuki, K., Ohtake, Y., Yamashita, H., 1998. Novel Beer-Spoilage *Lactobacillus* Species isolated from Breweries. J. Am. Soc. Brew. Chem. 56, 64–69.
- Giusto, C., Iacumin, L., Comi, G., Buiatti, S., Manzano, M., 2006. PCR-TTGE and RAPD-PCR Techniques to Analyze *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* isolated from Craft Beers. J. Inst. Brew. 112, 340–345.
- Giusto, C., Manzano, M., Bartolomeoli, I., Buiatti, S., Comi, G., 2005. Identificazione mediante PCR-DGGE dei Batteri Lattici alteranti la birra artigianale. Industria delle bevande. XXXIV, 207–210.
- Hammond J.R.M. 1996. Yeast genetics. In: Brewing Microbiology, 2nd Ed. (Eds. F.G. Priest and I. Campbell), pp.43–82, Chapman and Hale, London.
- Iserentant, T.E., 1994. Beers: recent technological innovations in brewing, in “Fermented beverage production” A.G.H. Lea, J.R., Piggot (Eds.), Blackie Academic and Professional, New York.

- Jespersen, L., van der Aa Kuhle, A., Petersen, K.M., 2000. Phenotypic and genetic diversity of *Saccharomyces* contaminants isolated from lager breweries and their phylogenetic relationship with brewing yeasts. *Int. J. Food Microbiol.* 60, 43–53.
- Jespersen, L., Jakobsen, M., 1996. Specific spoilage organisms in breweries and laboratory media for their detection. *Int. J. Food Microbiol.* 33, 139–155.
- Joubert, R., Brignon, P., Lehmann, C., Monribot, C., Gendre, F., Boucherie, H., 2000. Two-dimensional gel analysis of the proteome of lager brewing yeasts. *Yeast* 16, 511–522.
- Juvonen, R., Satokari, R., 1999. Detection of spoilage bacteria in beer by Polymerase Chain Reaction. *J. Am. Soc. Brew. Chem.* 7, 99–103.
- Kendall, N.T., 1995. Barley and Malt. In: Hardwick, W.A., *Handbook of brewing*. Ed. M. Dekker Inc., New York, 109–120.
- Kobi, D., Zugmeyer, S., Potier, S., Jaquet-Gutfreund, L., 2004. Two-dimensional protein map of an “ale”-brewing yeast strain: proteome dynamics during fermentation. *FEMS Yeast Research*. 5, 213–230.
- Kunze, W., 1999. Beer Production. In “Technology brewing and malting.” International Ed. VLB Berlin, 323–335.
- Manzano, M., Giusto, C., Bartolomeoli, I., Buiatti, S., Comi, G., 2005. Microbiological Analyses of Dry and Slurry Yeasts for Brewing. *J. Inst. Brew.* 111, 203–208.
- Mochaba, F.M., O'Connor-Cox, E.S.C., Axcell, B.C., 1997. A novel and practical yeast vitality method based on magnesium ion release. *J. Institute Brewing*. 103, 99–102.
- Naumova, E.S., Korshunova, I.V., Jespersen, L., Naumov, G.I., 2002. Molecular genetic identification of *Saccharomyces sensu strictu* strains from African sorghum beer. *FEMS Yeast Research*, 3, 177–184.
- Olesen, K., Felding, T., Gjermansen, C., Hansen, J., 2002. The dynamics of the *Saccharomyces carlsbergensis* brewing yeast transcriptome during a production-scale lager beer fermentation. *FEMS Yeast Research*. 2, 563–573.
- Rainieri, S., Kodama, Y., Kaneko, Y., Mikata, K., Nakao, Y., Ashikari T., 2006. Pure and mixed genetic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. *Appl. Environ. Microbiol.* 72, 3968–74.
- Satokari, R., Juvonen, R., Mallison, K., von Wright, A., Haikara, A., 1998. Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimeter microplate hybridization. *Int. J. Food Microbiol.* 45, 119–127.
- Stewart, R.J., Dowhanick, T.M., 1996. Rapid detection of lactic acid bacteria in fermenter samples using a Nested Polymerase Chain Reaction. *J. Am. Soc. Brew. Chem.* 54, 78–84.
- Suzuki, K., Sami, M., Ozaki, K., Yamashita, H., 2004. Nucleotide sequence identities of *hcrA* homologues and adjacent DNA regions identities in three species of beer-spoilage. *J. Instit. Brew.* 110, 276–283.
- Timke, M., Wang-Lieu, N.Q., Altendorf, K., Lipski, A., 2005. Community Structure and Diversity of Biofilms from a Beer Bottling Plant as Revealed Using 16S rRNA Gene Clone Libraries. *Appl. Environ. Microbiol.* 71, 6446–6452.
- Tompkins, T.A., Stewart, R., Savard, L., Russell, I., Dowhanick, T.M., 1996. RAPD-PCR Characterization of Brewery Yeast and Beer Spoilage Bacteria. *J. Am. Soc. Brew. Chem.* 54, 91–96.
- Tornai-Lehoczki, J., Dlauchy, D., 2000. Delimitation of brewing yeast strains using different molecular techniques. *Int. J. Food Microbiol.* 62, 37–45.
- Tsuchiya, Y., Kaneda, H., Kano, Y., Koshino, S., 1992. Detection of beer spoilage organisms by Polymerase Chain Reaction technology. *J. Am. Soc. Brew. Chem.* 50, 64–66.
- Vaughan, A., O'Sullivan, T., van Sinderen, D., 2005. Enhancing the Microbiological stability of malt and beer. A review. *J. Inst. of Brew.* 111, 355–371.
- Wightman, P., Quain, D.E., Meaden, P.G., 1996. Analysis of production brewing strains of yeast by DNA fingerprinting. *Lett. Appl. Microbiol.* 22, 90–94.
- Yamagishi, H., Otsuta, Y., Funahashi, W., Ogata, T., Sakai, K., 1999. Differentiation between brewing and non-brewing yeasts using a combination of PCR and RFLP. *J. Appl. Microbiol.* 86, 505–513.

Chapter 8

Other Fermentations

Christèle Humblot and Jean-Pierre Guyot

Abstract Fermented foods are staples for numerous consumers in many countries, especially in developing and emerging countries (DEC) where fermentation is often the only way to preserve food from microbial contaminations. Fermented foods from DEC are characterized by their wide diversity: they differ in the starting material (cereals, pulses, roots, vegetables, etc.), technology of production and the main microorganisms implicated in the fermentation process (lactic or acetic acid bacteria, yeasts, etc.). For cash crops like cocoa and coffee, fermentation is also an important step in the processing of cocoa beans and coffee cherries.

Compared to their large diversity, very few DEC fermentations were investigated using culture-independent methods. Mexican pozol and Korean kimchi can be considered model fermented foods for cereals and vegetables, respectively, to which culture-independent methods were applied for the first time. These techniques were also applied to other starchy foods, such as cassava dough, and more recently to investigate the microbial ecology of “rice black-vinegar” in Japan, cocoa and coffee fermentations. Such novel approaches are expected to improve the knowledge of the microbiology associated to these particular fermented foods.

1 Introduction

Fermented foods are essential components of the diet in many countries. Traditional know-how was transmitted and sometimes improved over centuries, contributing to the uniqueness of these foods and to the socio-cultural identity of their consumers. The fermentation is realized by microorganisms that play a key role in the physical, nutritional and organoleptic modification of the raw material. Researchers and the agro-food industry have paid close attention to these microorganisms for many years. Nevertheless, most of the studies focused on the fermented foods consumed in Western countries, mainly dairy products but also some vegetable-based foods and beverages like sauerkraut and wine, casting aside the numerous other fermented products that are staples or important economical resources for many consumers in developing and emerging countries (DEC). In Western countries food fermentations are often integrated in marketing strategies to construct nutritional claims, in

response to the increased attention paid by consumers to a healthy way of life, and to address specific organoleptic characteristics. This is, of course, a shortcut to emphasize the gap between the expectations of consumers from these countries and those of most developing countries and rural areas of emerging countries where, before other considerations, fermentation is still the unique and cheapest way to preserve food. The necessity of bringing safe food to DEC populations could be illustrated by the recurrent data from international organizations. It is estimated that 1.5 billion episodes of diarrhea occur annually in children under the age of 5, resulting in some 1.8 million deaths. Up to 70 percent of diarrheal episodes in DEC may be caused by food-borne contaminants (WHO 1997; Käferstein 2003). These public health considerations are very important, however, the economical importance of some raw materials must also be taken into account. For instance, important cash crops like cocoa and coffee are produced in DEC and a fermentation step is an important stage of the processing of cocoa beans and coffee cherries, which contributes to the organoleptic characteristics and facilitates the removal of the mucilage.

Without a doubt, culture-dependent methods brought to light important knowledge about those foods. However, our level of understanding must be deepened by using molecular approaches, mainly those that use culture-independent methods, for getting complementary or new information on previously studied fermentations, and to investigate other foods not yet or seldom studied. Molecular methods are essential to investigate the microbiota of fermented foods through community analysis, detection and characterization of yet-uncultured microorganisms, and to investigate the relationships between microbial diversity and functional properties. In this chapter such molecular approaches will be described for some fermented foods from DEC that differ in their raw materials and processing conditions.

2 Fermented Foods from Developing and Emerging Countries: A Brief Overview

The traditional fermented foods from DEC are very diverse. Table 8.1 lists some of the most common products prepared and consumed in various countries. A large variety of raw materials is used: cereals such as millet, maize, rice or sorghum; roots such as cassava or taro; pulses such as bean, chickpea or soybean, but also cocoa beans and coffee cherries. Fermented foods are prepared mainly in Africa and Asia, but also in Latin America and the Pacific Islands. They are consumed as beverages, gruels, porridges, soups, etc. and designated by specific names (Beuchat 1997). Sometimes different vernacular names can be used for the same or similar foods, generating some confusion in their identification. For example, *ogi* is also called *akamu* or *agidi*; moreover, it can be made of maize, millet or sorghum using similar processes (FAO 1993). Technologies used to make these foods are very diverse, resulting in complex changes of biochemical, sensory and nutritional characteristics. There can be one or several fermentation steps lasting from a few hours to several months, depending on the food. Fermentation is mostly natural (spontaneous) and,

therefore, noncontrolled but sometimes starter cultures are employed; for example, *Bacillus subtilis* causes the viscous appearance and texture of natto, a Japanese fermented food (Beuchat 1997). The microorganisms involved in the natural fermentations are more often lactic acid bacteria (LAB) and yeasts. They are responsible of the modification of the raw materials by their numerous lytic activities (proteolytic, amylolytic, lipasic, phytasic, etc.), and in the case of LAB, they allow the preservation of foods by inhibiting the development of spoilage and food-borne pathogens through acidification and bacteriocin production. However, the recognized importance of LAB and yeast should not dim the role of some fungi and *Bacillus* in producing some fermented foods or condiments. Fungi are responsible for soybean fermentation to produce tempeh and sufu (Kiers, et al. 2000; Han, et al. 2001), two important Asian fermented foods, whereas *Bacillus* species are responsible for the fermentation of African locust beans and soybean to produce soumbala and kinema, respectively (Sarkar, et al. 2002 ; Ouoba, et al. 2004). Soumbala is used as a flavoring agent in Burkina Faso, whereas kinema serves as a major source of protein in Nepal.

Most of the fermented foods presented here have been studied using a classical culture-dependent approach that selects a fraction of the existing microbial population. Microorganisms with important functional properties (e.g., production of antimicrobial compound, enzymes able to hydrolyze anti-nutritional factors, etc.) could, therefore, be underestimated by those which thrive on classical cultivation media or are numerically dominant in their natural environment. Moreover, the studies on fermented foods from DEC often describe the microbiota from only a few samples and do not take into account the possible variability between different production units and geographical areas. It is, therefore, sometimes difficult to draw general conclusions since the representativeness of results could be questioned. Classical microbiological methods are too heavy to implement when the microbiota of many samples coming from different production units and areas have to be analyzed. Food microbial ecology has known new developments since the advent of molecular methods which allowed a global approach without bias due to cultivation techniques (Ercolini 2004). To date, only a limited number of foods from DEC were studied by using such methods. The first works using culture-independent methods to investigate the microbiota of fermented foods from DEC were on maize-based foods, mainly pozol, a Mexican beverage made of a fermented maize dough, and kimchi, a Korean fermentation of vegetables. A few works present interesting results on cassava-, sorghum-, rice- and soybean-based fermented foods, and on cocoa and coffee.

3 Application of Molecular Methods to Cereal, Cassava and Soybean Fermentations

3.1 Maize-based Fermented Foods

Pozol (Table 8.1) is the first fermented food to which a culture-independent approach was applied to study the food microbiota in combination with the use of

Table 8.1 Examples of Traditional Fermented Foods Consumed in Various Countries

Raw material	Product	Country	Main microorganisms	Nature of product	Product use
African locust bean	Soumbala	Burkina Faso	<i>Bacillus spp.</i>	Solid	Condiment
African locust bean	Dawadawa	Nigeria	<i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i>	Solid	Side dish with rice
Cabbage/vegetables	Kimchi	Korea	LAB	Solid	Eaten with rice
Cassava	Chickwangué	Congo	LAB	Paste	Staple
Cassava	Gari	West Africa	LAB, yeasts	Flour	Staple
Cassava	Lafun	West Africa	LAB	Paste	Staple
Cassava	Cassava sour starch	Colombia, Brazil	LAB	Wet flour	Bread making
Chickpea and wheat	Khaman	India	LAB	Solid, cake-like	Breakfast
Chickpea and wheat	Dhokla	India	LAB	Spongy	Staple
Fish	Fish sauce	Asia	Bacteria	Liquid	Seasoning
Maize	Poto-poto	Congo	LAB	Paste	Breakfast, mush
Maize	Kenkey	Ghana	LAB, yeasts	Dough	Staple, stiff mush
Maize	Pozol	Mexico, Guatemala	LAB	Dough	“Food-beverage”
Maize, millet, sorghum	Ogi, mawè	West Africa	LAB, yeasts	Paste, porridge	Staple, mush
Maize, cassava	Banku	Ghana	LAB, yeasts	Dough	Staple
Maize, cassava	Mahewu	South Africa	LAB (<i>Lactobacillus delbrueckii</i>)	Liquid	Drink
Maize and finger millet malt	Togwa	East Africa	LAB	Liquid	Drink
Pearl millet	Bensaalga, koko	Burkina Faso, Ghana	LAB	Liquid	Mush
Rice	Puto	Philippines	LAB, other bacteria, <i>Saccharomyces</i>	Solid	Snack
Rice	Black rice vinegar	Japan	LAB, Acetic acid bacteria, yeasts, fungi	Liquid	Seasoning
Rice and bean	Dosai	India	<i>Lc. mesenteroides</i> , yeasts	Spongy, pancake-like	Breakfast
Rice and bean	Idli	India	LAB (<i>Lc. mesenteroides</i>), yeasts	Spongy, bread	Bread substitute

(continued)

Table 8.1 Examples of Traditional Fermented Foods Consumed in Various Countries (continued)

Raw material	Product	Country	Main microorganisms	Nature of product	Product use
Rice and soybeans or other cereals	Miso	Japan, China	LAB, yeasts	Paste	Soup base, seasoning
Sorghum	Hussuwa	Sudan	LAB	Dough	Staple
Sorghum and cassava	Burukutu	Nigeria	LAB, <i>Candida</i> spp., <i>Saccharomyces cerevisiae</i>	Liquid	Drink
Sorghum, maize	Pito	Nigeria	LAB, yeasts	Liquid	Drink
Soybean	Kinema	Nepal	<i>B. subtilis</i> , <i>Enterococcus faecium</i>	Solid	Staple
Soybean	Natto	Japan	<i>B. subtilis</i>	Moist, mucilaginous	Meat substitute
Soybean	Tempeh	Indonesia, Malaysia	<i>Rhizopus</i> spp.,	Solid	Meat substitute
Soybean	Sufu	China	<i>Rhizopus</i> , <i>Mucor</i> , <i>Actinomucor</i>	Creamy cheese-type	Like cheese
Soybean and wheat	Soy sauce	Asia	LAB, <i>Aspergillus oryzae</i> , <i>Zygosaccharomyces rouxii</i>	Liquid	Seasoning
Taro	Poi	Hawaii	LAB, <i>Candida vini</i> , <i>G. candidum</i>	Semi-solid	Side dish with fish, meat

culture-dependent methods in a polyphasic approach. Pozol is a traditional fermented maize dough prepared by Indians and Mestizos in Mexico and Guatemala. Nixtamalization is the first step during which kernels of white maize are cooked in the presence of lime. Thereafter, the grains are washed to remove the pericarps, coarsely ground, shaped into balls, wrapped in banana leaves and allowed to ferment at ambient temperature for two to seven or more days. The resulting fermented dough is suspended in water and drunk as a refreshing beverage. A wide variety of microorganisms, including yeasts, fungi and bacteria, were isolated from this spontaneous fermentation (Wacher, et al. 1993). LAB are by far the dominant microflora (Wacher, et al. 1993; Ampe, et al. 1999a; Ampe, et al. 1999b; ben Omar, et al. 2000a; Escalante, et al. 2001).

The abundance of the active microbial populations was evaluated by using a quantitative RNA hybridization method employing rRNA-targeted taxon-specific oligonucleotide probes (Ampe, et al. 1999a). The authors found that eukaryotes represented less than 6 percent of the microorganisms in pozol and, within bacteria, LAB were the most abundant microorganisms. Among LAB, there was a dominance of

Lactobacillus (20 percent to 65 percent) and *Streptococcus* (25 percent to 75 percent) while *Lactococcus* and *Leuconostoc* represented only a minor fraction of the bacterial community (Ampe, et al. 1999a; Ampe, et al. 1999b; ben Omar, et al. 2000a). The authors also investigated the spatial and temporal distribution of the microorganisms in pozol. They found more bacteria in the center of the ball than in the periphery, except for *Lactobacillus*, bifidobacteria and enterobacteria. Using this method, it was also possible to determine the succession of microflora. During pozol fermentation, the relative abundance of bifidobacteria, *Streptococcus* and *Lactobacillus* increased while it decreased for *Lactococcus* and *Leuconostoc* (Ampe, et al. 1999a; ben Omar, et al. 2000a). As for *Lb. fermentum*, it increased until 48 hours of fermentation and decreased at the end of the process (ben Omar, et al. 2000a). The results of rRNA quantification were compared to plate counts. LAB represented more than 90 percent of the active population, based on rRNA quantification, but only 10 percent to 50 percent by plate counts (Ampe, et al. 1999a, 1999b).

In parallel to the hybridization method, Ampe, et al. (1999b) used PCR-DGGE for community analysis. The PCR-DGGE fingerprints showed the presence of 18 bands for bacteria without major differences between the periphery and the center of the dough, and 20 faint bands at the periphery of the dough for eukaryotes (ben Omar, et al. 2000a). The major shift during fermentation of maize took place between 24 and 72 hours. The majority of the bands in the DGGE fingerprints of the bacterial community of pozol was sequenced and identified as *Acetobacter* sp., *B. subtilis*, *Lb. delbrueckii*, *Exiguobacterium acetylicum*, *Enterococcus saccharolyticus*, *Bifidobacterium minimum*, *Lb. casei* subgroup, *Streptococcus* sp., *Weissella-Leuconostoc* group and *Lb. plantarum-pentosus* group (Ampe, et al. 1999b; ben Omar, et al. 2000a). DNA extracted from each bacterial isolate from pozol was submitted to PCR-DGGE. They found that seven out of the 136 LAB strains isolated did not co-migrate with any of the bands identified in pozol samples, but conversely eight bands did not correspond to any of the isolates, in particular they failed to isolate the *Streptococcus* strain. These results illustrated that cultivation methods have limitations and molecular methods can fail in giving an exact picture of reality. This might be explained, in part, by biases due to PCR conditions and to preferential amplification of dominant and subdominant bacteria (Ercolini 2004). Another method, also based on a metagenomic approach, was used to study the pozol microbiota (Escalante, et al. 2001). Following the extraction of total DNA from the pozol, the 16S rRNA gene was amplified and cloned, and then sequenced and the corresponding bacteria were identified. The authors identified the species *L. lactis*, *St. suis*, *Lb. plantarum*, *Lb. casei*, *Lb. alimentarius*, *Lb. delbrueckii* and *Clostridium* sp. Consistently with other results (Ampe, et al. 1999a, 1999b; ben Omar, et al. 2000a) these species, except for *Clostridium* sp., were dominant as determined by 16S rRNA hybridization and sequencing of amplicons from DGGE bands. The pozol samples also contained *Bifidobacterium*, *Enterococcus* and enterobacteria, suggesting a fecal origin of some microorganisms (Ampe, et al. 1999b; ben Omar, et al. 2000a; Escalante, et al. 2001).

Different fermented maize-based foods – namely pozol, poto-poto and ogi from Mexico, Congo and Benin, respectively – (Ampe, et al. 2000) were compared on the basis of their DGGE profiles. To obtain poto-poto, maize is soaked (12 to 96 hours),

ground and sieved; then, water is added and the mixture is decanted (10 hours to 5 days) before putting the slurry in a bag and fermented for 10 hours to 7 days. Ogi is obtained after adding boiling water to the maize, soaking (24 hours), draining, grinding and sieving; then, water is added and the mix is fermented for two to seven days. Cluster analysis of DGGE profiles obtained for each type of food showed that microbial communities grouped in distinct clusters corresponding to a distinct type of foodstuff. Within a cluster similar richness and biodiversity indexes were shared. Interestingly, biodiversity and richness indexes were higher in pozol samples; the authors attributed these results to process conditions, e.g., higher initial pH of the product due to nixtamalization and some limited oxic conditions. Three DGGE bands corresponding to *Lb. plantarum*, *Lb. delbrueckii* and *Lb. fermentum* species were common to all the foods of different origins, suggesting that these species were particularly well adapted to the fermentation of maize (Ampe, et al. 2000). A similar approach was applied by Abriouel, et al. (2006) to poto poto and dégué (a pearl millet fermented dough from Burkina Faso). The authors performed community analysis by using temporal temperature gel electrophoresis (TTGE) to separate the PCR products obtained by amplification of the V3 region of the 16S rRNA gene from total DNA extracted according to three different methods, and concluded that the performance of DNA extraction largely depends on the food composition.

For the study of kenkey no culture-independent methods were applied, but isolates were characterized by applying molecular methods for identification and typing. Kenkey is a Ghanaian fermented maize dough, obtained by steeping the maize for 24 to 48 hours, followed by a milling step. The milled maize is reconstituted with water to form a stiff dough which is packed in troughs and left to ferment spontaneously for 48 to 72 hours. Hayford, et al. (1999) studied the diversity of *Lactobacillus* isolated from kenkey (Table 8.1) by random amplified polymorphic DNA (RAPD)-PCR. The authors showed that *Lb. fermentum* was dominant among other *Lactobacillus* spp. and had a large intraspecific diversity.

3.2 Sorghum-based Fermented Foods

Kunene, et al. (2000) investigated the lactic acid microflora in a sorghum powder and corresponding fermented cooked porridge made in South Africa. They used a similar polyphasic approach to that of Hayford, et al. (1999) for kenkey, except that diversity among the sorghum LAB isolates was analyzed by using both total soluble proteins and amplified fragment length polymorphism (AFLP). The authors found that the majority of the LAB belonged to *Lactobacillus* and *Leuconostoc* genera and, to a less extent, to *Lactococcus* and *Pediococcus* genera. The analysis of diversity by AFLP showed that the dominant *Lb. plantarum* strains were originated from the sorghum powder while the dominant *Lc. mesenteroides* strains were originated from both the sorghum powder and the household environment.

In a similar approach Yousif, et al. (2005) studied the *Enterococcus* population of husuwa, a semi-solid sorghum-based fermented food (Table 8.1). Using RAPD

fingerprinting, 16S rRNA sequencing and RFLP analysis, the authors found that all the *Enterococcus* isolates were *E. faecium* species with a great genetic diversity (Yousif, et al. 2005).

3.3 Cassava-based Fermented Foods

Three cassava-based fermented products have been studied, namely the cassava sour starch from Colombia, a fermented cassava dough and gari from the Congo (Table 8.1) using a combination of culture-independent methods and cultivation techniques.

Cassava sour starch processing starts by washing and peeling the roots, that are then grated in rotors with perforated blades. The starch present in the suspension is separated by sieving from the pulp or bran; the slurry from sieving is allowed to settle and particles of fibers and other fine materials that are not removed by sieving are separated from the slurry. The wet starch is then passed through a series of tanks in which it remains for several weeks (ben Omar, et al. 2000b). Gari is prepared by grating the cassava root, followed by dewatering, fermentation for two days at ambient temperature and roasting of the fermented mash (Kostinek, et al. 2005).

The microbiota of cassava sour starch was studied by RAPD-PCR, plasmid profiling, hybridization using rRNA phylogenetic probes and partial 16S rRNA gene sequencing. A large diversity of bacteria and yeasts was described, namely *Lb. manihotivorans*, *Lb. plantarum*, *Lb. casei*, *Lb. hilgardii*, *Lb. buchneri*, *Lb. fermentum*, *Lb. perolans*, *Lb. brevis*, *Lc. mesenteroides*, *Pediococcus* sp., a low number of *B. cereus*, *Galactomyces geothricum*, *Issatchenkia* sp. and *Candida ethanolica* (ben Omar, et al. 2000b; Lacerda, et al. 2005). However, the most frequently isolated species were *Lb. plantarum* and *Lb. manihotivorans* with a large molecular diversity as revealed by RAPD analysis (ben Omar, et al. 2000b). Using PCR-DGGE followed by sequencing of the most intense bands, Ampe, et al. (2001) confirmed that LAB were the dominant organisms, mainly close relatives of *L. lactis*, *Streptococcus* sp., *E. saccharolyticus*, *Lb. plantarum*, *Lb. panis*, *Lc. mesenteroides* and *Lc. citreum* (Ampe, et al. 2001). As the PCR products from *Lb. manihotivorans* and *L. lactis* co-migrate, a complementary analysis using hybridization of 16S rRNA with phylogenetic probes was necessary to detect the presence of the species *Lb. manihotivorans* (Ampe, et al. 2001). The use of molecular methods showed that in sour cassava starch, *Lb. manihotivorans* could represent up to 20 percent of total LAB (Ampe 2000; Ampe, et al. 2001). It is, therefore, not surprising that in a previous study on the amylolytic LAB in this Colombian niche product, *Lb. manihotivorans* strains were isolated for the first time (Morlon-Guyot, et al. 1998). In this case the isolation of a new microorganism preceded its detection by molecular tools which, in turn, enabled scientists to confirm its status as dominant LAB. In contrast, another study using PCR-DGGE and sequencing of 16S rRNA genes allowed detecting the presence of this bacterium in a Congolese fermented cassava dough, whereas cultivation techniques failed in isolating it (Miambi, et al. 2003).

As for the African fermented cassava dough, Miambi, et al. (2003) coupled the two approaches: they compared the results obtained by using PCR- DGGE and classical culture-dependent methods, from samples submitted or not to an enrichment culture step. It appeared that DGGE profiles of total DNA of cassava dough exhibited 10 distinguishable bands. As it could be expected, DGGE fingerprints of bacteria recovered from enrichment cultures of fermented dough gave variable profiles containing fewer bands. Bands corresponding to five bacterial species detected by direct PCR-DGGE of total DNA from cassava dough were also observed in DGGE patterns from enrichment cultures. Eighteen strains were isolated from cultures selected on the basis of their DGGE banding patterns. The sequence of DGGE bands revealed that representative bacteria of fermented cassava dough were *Lactobacillus* and *Pediococcus* species, as well as species of *Clostridium*, *Propionibacterium* and *Bacillus*. Some *Lactobacillus* species detected in dough samples by sequence analysis of DGGE bands were not recovered in any of the five culture media and conditions used. On the contrary, some species recovered as pure cultures from enrichments were not detected by direct DGGE analysis of total bacterial DNA from cassava dough (Miambi, et al. 2003). These results illustrate the same limitations previously described for pozol.

The study of LAB diversity in gari by Kostinek, et al (2005) was made by using phenotypic tests and genotypic methods such as RAPD-PCR, DNA-DNA hybridization or sequencing of the 16S rRNA genes. One-hundred-thirty-nine strains isolated from fermented cassava were identified. *Lb. plantarum* was the most abundant species (54.6 percent of isolates), followed by *Lc. fallax* (22.3 percent) and *Lb. fermentum* (18.0 percent). Moreover, *Lb. brevis*, *Lc. pseudomesenteroides* and *W. paramesenteroides* were sporadically isolated.

3.4 Miscellaneous

Molecular methods were applied to some soybean and rice fermented foods (Haruta, et al. 2006; Inatsu, et al. 2006; Suezawa, et al. 2006).

Inatsu, et al. (2006) investigated the diversity of *B. subtilis* strains isolated from thua nao, a traditional Thai fermented soybean food, by RAPD-PCR fingerprinting. They found that the strains were divided into 19 types, including a type with the same pattern as a Japanese natto-producing strain (Inatsu, et al. 2006). In Japan, Suezawa, et al. (2006) analyzed the sequences of the D1D2 domain of the 26S ribosomal RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2 (ITS sequence) of the miso and soy sauce fermentation yeasts, *C. etchellsii* and *C. versatilis*. They found that those molecular methods were rapid and precise compared with the physiological method for the identification and typing of these two species (Suezawa, et al. 2006).

PCR-DGGE based on the 16S rRNA gene was applied to a traditional Japanese fermentation process to produce "rice black vinegar" (kome-kurozu), in which the conversion of rice starch into acetic acid proceeds in a ceramic pot inoculated with

rice koji. Pot vinegar fermentation offers an interesting field of study, since three microbiological processes involving yeast, fungi and bacteria occur simultaneously or sequentially. The fungal DGGE profiles during the pot vinegar fermentation process indicated that the transition from *Aspergillus oryzae* to *Saccharomyces* sp. took place at the initial stage at which alcohol production was observed. The early stage was characterized by the coexistence of *Saccharomyces* sp. and LAB. Most of the bacterial DGGE bands related to LAB were replaced by bands derived from *Lb. acetotolerans* and *Acetobacter pasteurianus* when acetic acid started to accumulate. Similar to other studies, among the bacteria isolated at the early stage, some species differed from those detected by DGGE (Haruta, et al. 2006).

4 Kimchi: A Case Study for Molecular Ecology of Vegetable Fermentation

Kimchi (Table 8.1) is a traditional Korean fermented food prepared by trimming oriental cabbage (or other vegetables), brining, blending with various spices (including garlic, ginger and hot pepper), and fermentation (Cheigh, et al. 1994). Microbial ecology of this food was investigated by both culture-dependent and -independent methods.

First works were based on the use of molecular methods to identify the microorganisms isolated from kimchi, often in parallel with phenotypic characterization. For example, Choi, et al. (2003) have shown through 16S rRNA sequencing that 68 percent of the 120 LAB isolated from kimchi were *Lc. citreum*. The other dominant species were *Lb. sakei*, *Lb. curvatus*, *Lb. brevis* and *W. confusa*-like microorganisms. *Lc. citreum* was dominant during the early and mid phase of kimchi fermentations while the other bacteria were found during the later stages (Choi, et al. 2003). In another study on microbial diversity using different methods based on amplified 16S rRNA gene-based restriction enzyme assay, Cho, et al. (2006) determined that the 970 bacteria isolated from kimchi belong to 15 species of the genera *Lactobacillus*, *Leuconostoc* and *Weissella*. They investigated the influence of fermentation temperature and showed that the *Leuconostoc* species was favored during the preliminary two-day incubation at 15 °C, while *W. koreensis* predominated in the second fermentation phase realized at -1 °C. When the preliminary incubation period was realized at 10 °C for four days, only *W. koreensis* grew rapidly at the beginning of the process (Cho et, al. 2006).

The use of 16S rRNA gene sequencing and DNA-DNA hybridization on LAB isolated from kimchi also enabled the identification of several novel species, namely *Lc. kimchii* (Kim, et al. 2000), *Lb. kimchii* (Yoon, et al. 2000), *W. kimchii* (Choi, et al. 2002), *W. koreensis* (Lee, et al. 2002) and *Lc. inhae* (Kim, et al. 2003).

Further studies on the microbiota of kimchi used culture-independent approaches. The examination of 16S rRNA gene clone libraries using amplified ribosomal DNA restriction analysis (ARDRA) and sequencing showed that *W. koreensis* was the only species found in all kimchi samples from five manufacturers. It was generally the most abundant, followed by *Leuconostoc* and *Lactobacillus* genera (Kim, et al. 2005).

Using PCR-DGGE for microbial community analysis, Lee, et al. (2005) found that kimchi samples fermented at 10 °C or 20 °C for 30 or 20 days, respectively, exhibited up to 12 bands. Their sequencing revealed that the main microorganisms responsible for kimchi fermentation were *W. confusa*, *Lc. citreum*, *Lb sakei* and *Lb. curvatus*. Bands corresponding to *W. confusa* and *Lc. citreum* remained present throughout the fermentation process, indicating their importance in kimchi fermentation. *Lb. sakei* and *Lb. curvatus* were also identified as significant components of the bacterial community. The authors found that the microorganisms involved in the initial stage of kimchi fermentation were different from those in the late stage. For example, an uncultured bacterium present initially disappeared during the fermentation and *L. lactis* subsp. *lactis* only appeared after two days of incubation. The fermentation at both temperatures gave some slight differences: *Lc. gelidum* and *Serratia marcescens* were only found in the kimchi fermented at 10 °C (Lee, et al. 2005).

The identification of pathogenic microorganisms was also realized on commercially available kimchi using a multiplex PCR designed for the simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes*. The authors found that one out of four samples were contaminated with *Listeria monocytogenes* and none with the other pathogens (Park, et al. 2006).

The genome-probing microarray (GPM) introduced a genomic technology in the study of microbial ecology of DEC foods. GPM allows the profiling of a microbial community based on whole-genome DNA-DNA hybridization. As a probe the GPM contained genomic DNA isolated from 149 different strains of LAB. When compared to other culture-independent methods its main advantage is independence on PCR amplification (Bae, et al. 2005). The authors found that the number of positive signals in the late phase of commercial kimchi samples (71 to 99 species) was higher than in the early phase samples (28 to 45 species). As the fermentation progressed, the most abundant species belonged to the genus *Weissella* and *Leuconostoc* and, to a lesser extent, to the genus *Lactobacillus*, *Enterococcus*, *Pediococcus* and *Lactococcus*. A few *Streptococcus* species were also present and no *Bifidobacterium* were found in kimchi. Most species in the genus *Weissella* were present in the late kimchi phase of fermentation. In the same study, the authors compared the GPM to PCR-DGGE analysis. They found only nine different microorganisms from DGGE analysis whereas GMP analysis revealed the presence of 99 microorganisms, therefore establishing itself as a powerful tool to investigate food microbiota without bias due to limitations of PCR-based methods. The use of GPM confirmed the presence of species usually found in kimchi, such as those belonging to the genera *Weissella* or *Leuconostoc*, and enabled the detection of species not usually described in kimchi fermentation, such as *Pediococcus* (Choi, et al. 2003, 2006).

Different works published on kimchi show some discrepancies regarding population structure and dynamics. These works were based either on kimchi made at laboratory scale or on commercial kimchi purchased from different manufacturers. Furthermore, "kimchi" is a generic term for a group of fermented vegetable foods

in Korea, for which more than 100 types of vegetables can be used. Therefore, the variability in results is not surprising and could be explained by differences in the processing conditions and the vegetables used.

5 Cocoa and Coffee Fermentation

5.1 Cocoa Fermentation

Cocoa beans are derived from the fruit pods of the tree *Theobroma cacao*. Each fruit pod contains 30 to 40 beans embedded in a mucilaginous pulp. Raw cocoa has an astringent, unpleasant taste and flavor and must be fermented, dried and roasted to obtain the characteristic cocoa flavor and taste (Beuchat 1997). Several different fermentation systems are used around the world; of these heap and box fermentations are the most commonly used (Baker, et al. 1994). As for “rice black vinegar,” a complex pattern of fermentation involves the succession of yeasts, LAB and acetic acid bacteria. Several studies investigated the cocoa bean fermentations using culture-dependent methods (Ardhana, et al. 2003; Lagunes, et al. 2007). The microbial ecology of cocoa fermentation has been newly investigated using a polyphasic approach combining molecular methods, including PCR-DGGE and chromosome length polymorphism (CLP), with culture-dependent methods (Jespersen, et al. 2005; Nielsen, et al. 2005; Camu, et al. 2007; Nielsen, et al. 2007).

The yeast and bacterial populations from tray and traditional fermentations in Ghana were investigated on samples collected at 12-hour intervals during 96- to 144-hour fermentation. Yeasts, LAB, acetic acid bacteria (AAB) and *Bacillus* spp. were enumerated and identified using phenotypic and molecular methods, and further investigated using PCR-DGGE (Jespersen, et al. 2005; Nielsen, et al. 2005; Camu, et al. 2007; Nielsen, et al. 2007). A microbiological succession was observed during the fermentations. At the onset of the fermentation yeasts were the dominating microorganisms. LAB became dominant after 12 to 24 hours up to the end of the fermentation, and AAB reached high counts in the mid-phase (Nielsen, et al. 2005).

With regard to yeasts, *Hanseniaspora guilliermondii* and *Pichia membranifaciens* were dominant in both types of fermentation (Jespersen, et al. 2005; Nielsen, et al. 2005, 2007). A number of other yeast species – *C. krusei*, *Pichia kluyveri*, *Trichosporon asahii* and *S. cerevisiae* – were found depending on the study (Jespersen, et al. 2005; Nielsen, et al. 2005). For dominant yeasts intraspecies variations were examined by CLP using pulsed-field gel electrophoresis, showing that several different strains were involved in the fermentations (Jespersen, et al. 2005). In general, the culture-based findings were confirmed using PCR-DGGE. Nevertheless, the use of PCR-DGGE revealed the presence of *C. zemplinina* that were not found using culture-dependent methods. On the other hand, *T. asahii* yielded only faint bands in DGGE, despite the fact that it was detected using

culture-based methods. This is explained by the fact that the targeted region of the 26S rRNA gene was poorly amplified in *T. asahii*, whereas all other investigated isolates were amplified efficiently (Nielsen, et al. 2005). On one occasion three putatively undescribed yeast species were isolated (Nielsen, et al. 2007). The origin of the yeasts was also researched. Isolates of *C. krusei*, *Pi. membranifaciens*, *H. guilliermondii*, *T. asahii* and *Rhodotorula glutinis* were found on the surface of the cocoa pods and, in some cases, on the production equipment whereas the origin of other yeasts (e.g., *S. cerevisiae*) was not elucidated (Jespersen, et al. 2005). As for bacteria, *Bacillus* spp. were only detected during heap fermentations where they reached high numbers during the later stages of fermentation (Nielsen, et al. 2007). Four main clusters, namely *Lb. plantarum*, *Lb. fermentum*, *Lc. pseudomesenteroides*, and *E. casseliflavus*, were identified among the LAB isolated (Camu, et al. 2007). Several other LAB, including *Lc. pseudoficulneum*, *P. acidilactici* and the genus *Weissella*, were also detected (Nielsen, et al. 2007). Only four clusters were found among the AAB identified: *Acetobacter pasteurianus*, *A. syzygii*-like bacteria, and two small clusters of *A. tropicalis*-like bacteria (Camu, et al. 2007; Nielsen, et al. 2007). The culture-based findings differed slightly from the DGGE outcomes. For example, DGGE indicated that *Lc. pseudoficulneum* plays a more important role during the fermentation of cocoa than expected from the culture-based findings as it yielded a strong band in most DGGE fingerprints. A newly proposed species of LAB ("*Weissella ghanaensis*") was detected by PCR-DGGE in heap fermentations and only occasionally isolated. Also, two new species of *Acetobacter* – tentatively named "*Acetobacter senegalensis*" (*A. tropicalis*-like) and "*Acetobacter ghanaensis*" (*A. syzygii*-like) – were isolated (Camu, et al. 2007).

The authors concluded that the fermentation of cocoa beans is a very inhomogeneous process with great variations in both yeast counts and species composition. Cluster analysis of the DGGE fingerprints revealed that the variations seem to depend especially on the processing procedure, but also the season and the post-harvest storage are likely to influence the yeast counts and the species composition (Jespersen, et al. 2005; Nielsen, et al. 2005, 2007).

5.2 Coffee Fermentation

Commercial coffee beans belong to the species *Coffea arabica* and *Coffea canephora* var. *robusta*. To separate beans from pulp, coffee is processed by dry or wet method. The dry method is mainly used for *robusta* coffee. In wet processing the ripe coffee cherries are pulped, followed by fermentation and drying. Coffee fermentation removes the pectineous mucilage adhering to coffee beans. Yeasts involved in *Coffea arabica* fermentation in Tanzania were characterized by genotyping of the isolates using ITS-PCR and sequence analysis of the D1/D2 domain of the 26S rRNA gene. DGGE was performed on PCR-amplified 26S rRNA gene to detect yeasts from coffee samples (Masoud, et al. 2004). This work showed that *Pi. kluyveri* was dominant during fermentation and drying; *H. uvarum* was dominant during fermentation whereas numerous *Pi. anomala* were found during drying.

S. cerevisiae and *C. xestobii* were not isolated, but they were detected by DGGE. The authors found a good agreement between the sequence analysis of the D1/D2 domain of the 26S rRNA gene and sequencing of the DGGE bands.

6 Perspectives

This examples in this survey illustrate what was accomplished by using molecular methods to study traditional fermented foods from DEC. A reasonable combination of molecular methods, through a metagenomic approach, and culture-dependent methods would offer better chances to improve our knowledge of the microbial ecology of such foods. Within the molecular tool box, PCR-DGGE is one of the most popular methods for community analysis. It is considered relatively inexpensive and easy to use, and might easily be applied by researchers in DEC countries where research resources are scarce. Alternatively, TTGE could also be easier to implement since no chemical denaturing gradient is necessary. One of the main benefits of using culture-independent methods is the potential to investigate microbial diversity in numerous samples, thereby eliminating the need for culture-dependent techniques that necessitate huge amounts of Petri dishes and cultivation medium. However, it will still be necessary to isolate pure strains, to select them for their interesting biochemical characteristics and also to develop appropriate starter cultures if the traditional processes are upgraded to a larger and safer scale of production.

In general, investigations into the microbial ecology of foods in the tropical and sub-tropical world reveal that many identified microorganisms are also found in traditional Western fermentations. For instance, among the most common microorganisms, LAB like *Lb. plantarum* and *Lb. fermentum* and yeasts like *S. cerevisiae* are ubiquitous microorganisms, which are repeatedly isolated from fermentations around the world, sometimes sharing the same “fermentation pot” (like in African sorghum or maize-based beers). Why does this happen? This is a difficult question to address. Over centuries, trade between continents, invasions, exchange of seeds or introduction of plants from one continent to another – like cassava and maize introduced by the Portuguese from Latin America to Africa in the 16th century – or dissemination through bird migrations among other reasons could have contributed to spread these food microorganisms throughout the world. What would a phylogeographical study reveal about the migratory scheme of one ubiquitous species? In contrast, investigations on traditional fermented foods, as described here, indicate that some microorganisms could be specific to their food niche, such as *Lb. manihotivorans* (Morlon-Guyot, et al. 1998), *W. kimchii* (Choi, et al. 2002), *W. koreensis* (Lee, et al. 2002), etc., but are they really as specific as they first appear (e.g., only found in those niches) or was their isolation facilitated because they were dominant in their food niche? That question is illustrated by the case of *Lb. manihotivorans* which was dominant in the Colombian cassava sour starch and easily isolated from that product, but never isolated elsewhere or only detectable in a Congolese cassava fermented dough by a culture-independent method. However, questions remain regarding the reason for their dominance in specific areas and foods, and why they

do not disseminate around the world like other ubiquitous microorganisms. Therefore, tracking such “specific” microorganisms in different foods around the world by using molecular approaches is an interesting ecological issue to address.

References

- Abriouel, H., ben Omar, N., Lopez, R.L., Martinez-Canamero, M., Keleke, S., Galvez, A., 2006. Culture-independent analysis of the microbial composition of the African traditional fermented foods *poto poto* and *degue* by using three different DNA extraction methods. *Int. J. Food Microbiol.* 111, 228–233.
- Ampe, F., 2000. Design and evaluation of a *Lactobacillus manihotivorans* species-specific rRNA-targeted hybridization probe and its application to the study of sour cassava fermentation. *Appl. Environ. Microbiol.* 66, 2224–6.
- Ampe, F., ben Omar, N., Guyot, J.P., 1999a. Culture-independent quantification of physiologically active microbial groups in fermented foods using rRNA-targeted oligonucleotide probes: application to pozol, a Mexican lactic acid fermented maize dough. *J. Appl. Microbiol.* 87, 131–40.
- Ampe, F., ben Omar, N., Moizan, C., Wachter, C., Guyot, J.P., 1999b. Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Appl. Environ. Microbiol.* 65, 5464–73.
- Ampe, F., Miambi, E., 2000. Cluster analysis, richness and biodiversity indexes derived from denaturing gradient gel electrophoresis fingerprints of bacterial communities demonstrate that traditional maize fermentations are driven by the transformation process. *Int. J. Food Microbiol.* 60, 91–7.
- Ampe, F., Sirvent, A., Zakhia, N., 2001. Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization. *Int. J. Food Microbiol.* 65, 45–54.
- Ardhana, M.M., Fleet, G.H., 2003. The microbial ecology of cocoa bean fermentations in Indonesia. *Int. J. Food Microbiol.* 86, 87–99.
- Bae, J.W., Rhee, S.K., Park, J.R., Chung, W.H., Nam, Y.D., Lee, I., Kim, H., Park, Y.H., 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. *Appl. Environ. Microbiol.* 71, 8825–35.
- Baker, D.M., Tomlins, K.I., Gay, C., 1994. Survey of Ghanaian cocoa farmer fermentation practices and their influence on cocoa flavor. *Food Chemistry*. 51, 425–431.
- ben Omar, N., Ampe, F., 2000a. Microbial community dynamics during production of the Mexican fermented maize dough pozol. *Appl. Environ. Microbiol.* 66, 3664–73.
- ben Omar, N., Ampe, F., Raimbault, M., Guyot, J.P., Tailliez, P., 2000b. Molecular diversity of lactic acid bacteria from cassava sour starch (Colombia). *Syst. Appl. Microbiol.* 23, 285–91.
- Beuchat, L.R., 1997. Traditional fermented foods. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. (Eds.), *Food Microbiology - fundamentals and frontiers*. ASM press, Washington DC, 629–648.
- Camu, N., De Winter, T., Verbrugghe, K., Cleenwerck, I., Vandamme, P., Takrama, J.S., Vancanneyt, M., De Vuyst, L., 2007. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. *Appl. Environ. Microbiol.* 73, 1809–24.
- Cheigh, H.S., Park, K.Y., 1994. Biochemical, microbiological, and nutritional aspects of kimchi (Korean fermented vegetable products). *Crit. Rev. Food Sci. Nutr.* 34, 175–203.
- Cho, J., Lee, D., Yang, C., Jeon, J., Kim, J., Han, H., 2006. Microbial population dynamics of kimchi, a fermented cabbage product. *FEMS Microbiol. Lett.* 257, 262–7.
- Choi, H.J., Cheigh, C.I., Kim, S.B., Lee, J.C., Lee, D.W., Choi, S.W., Park, J.M., Pyun, Y.R., 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. *Int. J. Syst. Evol. Microbiol.* 52, 507–11.

- Choi, I.K., Jung, S.H., Kim, B.J., Park, S.Y., Kim, J., Han, H.U., 2003. Novel *Leuconostoc citreum* starter culture system for the fermentation of kimchi, a fermented cabbage product. *Antonie Van Leeuwenhoek* 84, 247–53.
- Ercolini, D., 2004. PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *J. Microbiol. Meth.* 56, 297–314.
- Escalante, A., Wachter, C., Farres, A., 2001. Lactic acid bacterial diversity in the traditional Mexican fermented dough pozol as determined by 16S rDNA sequence analysis. *Int. J. Food Microbiol.* 64, 21–31.
- FAO, 1993 [Maize in human nutrition] *Le maïs dans l'alimentation humaine*. Vol. 25. FAO, Rome.
- Han, B.Z., Rombouts, F.M., Nout, M.J., 2001. A Chinese fermented soybean food. *Int. J. Food Microbiol.* 65, 1–10.
- Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., Ishii, M., Igarashi, Y., 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *Int. J. Food Microbiol.* 109, 79–87.
- Hayford, A.E., Petersen, A., Vogensen, F.K., Jakobsen, M., 1999. Use of conserved randomly amplified polymorphic DNA (RAPD) fragments and RAPD pattern for characterization of *Lactobacillus fermentum* in Ghanaian fermented maize dough. *Appl. Environ. Microbiol.* 65, 3213–21.
- Inatsu, Y., Nakamura, N., Yuriko, Y., Fushimi, T., Watanasiritum, L., Kawamoto, S., 2006. Characterization of *Bacillus subtilis* strains in Thua nao, a traditional fermented soybean food in Northern Thailand. *Lett. Appl. Microbiol.* 43, 237–42.
- Jespersen, L., Nielsen, D.S., Honholt, S., Jakobsen, M., 2005. Occurrence and diversity of yeasts involved in fermentation of West African cocoa beans. *FEMS Yeast Res.* 5, 441–53.
- Käferstein, F.K., 2003. Food safety as a public health issue for developing countries. In: Laurian, J.U. (Ed.), *Food safety in food security and food trade. Focus, 2020 vision for food, agriculture and the environment*, vol 10. IFPRI, 2–17.
- Kiers, J.L., Nout, M.J., Rombouts, F.M., 2000. In vitro digestibility of processed fermented soy bean, cowpea and maize. *J. Sc. Food Agri.* 80, 1325–1331.
- Kim, B., Lee, J., Jang, J., Kim, J., Han, H., 2003. *Leuconostoc inhae* sp. nov., a lactic acid bacterium isolated from kimchi. *Int. J. Syst. Evol. Microbiol.* 53, 1123–6.
- Kim, J., Chun, J., Han, H.U., 2000. *Leuconostoc kimchii* sp. nov., a new species from kimchi. *Int. J. Syst. Evol. Microbiol.* 50 Pt 5, 1915–9.
- Kim, M., Chun, J., 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. *Int. J. Food Microbiol.* 103, 91–6.
- Kostinek, M., Specht, I., Edward, V.A., Schillinger, U., Hertel, C., Holzapfel, W.H., Franz, C.M., 2005. Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. *Syst. Appl. Microbiol.* 28, 527–40.
- Lacerda, I.C., Miranda, R.L., Borelli, B.M., Nunes, A.C., Nardi, R.M., Lachance, M.A., Rosa, C.A., 2005. Lactic acid bacteria and yeasts associated with spontaneous fermentations during the production of sour cassava starch in Brazil. *Int. J. Food Microbiol.*
- Lagunes Gálvez, S., Loiseau, G., Paredes, J.L., Barel, M., Guiraud, J.P., 2007. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. *Int. J. Food Microbiol.* 114, 124–130.
- Lee J.S., Heo G.Y., Lee J.W., Oh Y.J., Park J.A., Park Y.H., Pyun Y.R., Ahn J.S., 2005. Analysis of kimchi microflora using denaturing gradient gel electrophoresis. *Int J Food Microbiol.* 102, 143–50.
- Lee, J.S., Lee, K.C., Ahn, J.S., Mheen, T.I., Pyun, Y.R., Park, Y.H., 2002. *Weissella koreensis* sp. nov., isolated from kimchi. *Int. J. Syst. Evol. Microbiol.* 52, 1257–61.
- Masoud, W., Cesar, L.B., Jespersen, L., Jakobsen, M., 2004. Yeast involved in fermentation of *Coffea arabica* in East Africa determined by genotyping and by direct denaturing gradient gel electrophoresis. *Yeast* 21, 549–56.
- Miambi, E., Guyot, J.P., Ampe, F., 2003. Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 82, 111–20.

- Morlon-Guyot, J., Guyot, J.P., Pot, B., Jacobe de Haut, I., Raimbault, M., 1998. *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolyzing lactic acid bacterium isolated from cassava sour starch fermentation. *Int. J. Syst. Bacteriol.* 48, 1101–1109.
- Nielsen, D.S., Honholt, S., Tano-Debrah, K., Jespersen, L., 2005. Yeast populations associated with Ghanaian cocoa fermentations analyzed using denaturing gradient gel electrophoresis (DGGE). *Yeast* 22, 271–84.
- Nielsen, D.S., Teniola, O.D., Ban-Koffi, L., Owusu, M., Andersson, T.S., Holzapfel, W.H., 2007. The microbiology of Ghanaian cocoa fermentations analyzed using culture-dependent and culture-independent methods. *Int. J. Food Microbiol.* 114, 168–86.
- Ouoba, L.I., Diawara, B., Amoa-Awua, W., Traore, A.S., Moller, P.L., 2004. Genotyping of starter cultures of *Bacillus subtilis* and *Bacillus pumilus* for fermentation of African locust bean (*Parkia biglobosa*) to produce Soumbala. *Int. J. Food Microbiol.* 90, 197–205.
- Park, Y.S., Lee, S.R., Kim, Y.G., 2006. Detection of *Escherichia coli* O157:H7, *Salmonella* spp., *Staphylococcus aureus* and *Listeria monocytogenes* in kimchi by multiplex polymerase chain reaction (mPCR). *J. Microbiol.* 44, 92–7.
- Sarkar, P.K., Hasenack, B., Nout, M.J., 2002. Diversity and functionality of *Bacillus* and related genera isolated from spontaneously fermented soybeans (*Indian Kinema*) and locust beans (African Soumbala). *Int. J. Food Microbiol.* 77, 175–86.
- Suezawa, Y., Kimura, I., Inoue, M., Gohda, N., Suzuki, M., 2006. Identification and typing of miso and soy sauce fermentation yeasts, *Candida etchellsii* and *C. versatilis*, based on sequence analyses of the D1D2 domain of the 26S ribosomal RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2. *Biosci. Biotechnol. Biochem.* 70, 348–54.
- Wacher, C., Canas, A., Cook, P.E., Barzana, E., Owens, J.D., 1993. Sources of microorganisms in pozol, a traditional Mexican fermented maize dough. *World J. Microbiol. Biotechnol.* 9, 269–274.
- WHO, 1997. Food safety and food-borne diseases. *World Health Stat Quarterly*, N° 1/2, p.50.
- Yoon, J.H., Kang, S.S., Mheen, T.I., Ahn, J.S., Lee, H.J., Kim, T.K., Park, C.S., Kho, Y.H., Kang, K.H., Park, Y.H., 2000. *Lactobacillus kimchii* sp. nov., a new species from kimchi. *Int. J. Syst. Evol. Microbiol.* 50 Pt 5, 1789–95.
- Yousif, N.M., Dawyndt, P., Abriouel, H., Wijaya, A., Schillinger, U., Vancanneyt, M., Swings, J., Dirar, H.A., Holzapfel, W.H., Franz, C.M., 2005. Molecular characterization, technological properties and safety aspects of enterococci from ‘Hussuwa,’ an African fermented sorghum product. *J. Appl. Microbiol.* 98, 216–228.

Chapter 9

Probiotics: Lessons Learned from Nucleic Acid-based Analysis of Bowel Communities

Rodrigo Bibiloni, Christophe Lay, and Gerald W. Tannock

Abstract The majority of probiotics are administered as dietary supplements either in milk-based foods, or as tablets and capsules. Consumption of probiotic products is usually aimed at improving the general health of the consumer, although a recent trend has been to use them in ameliorating the condition of sufferers of specific diseases that affect the bowel. Traditionally, it has been believed that probiotics benefit the consumer by altering the composition of the bacterial community that inhabits the bowel. However, evidence to support this view have been difficult to obtain since the majority of the bowel inhabitants have not yet been cultivated under laboratory conditions. Culture-independent, nucleic acid-based analytical methods targeting the 16S rRNA gene have, therefore, become an essential adjunct to traditional bacteriological approaches in studies of the impact of probiotic consumption on the bowel ecosystem. Commonly used analytical methods that are used in probiotic studies are described and evaluated critically in this chapter. Examples of knowledge that have been gained through use of these analytical methods is presented.

1 Introduction

Human large bowel contents and feces contain about 10^{11} bacterial cells per gram (wet weight) and bacterial cells comprise about 50 percent of fecal mass (Suau, et al. 1999). Four bacterial phyla are represented (Firmicutes [gram positive], Bacteroidetes [gram negative], Actinobacteria [gram positive], and Proteobacteria [gram negative]), and three groups, still phylogenetically broad and each containing many genera and species, are numerically dominant in the feces of healthy humans (*Clostridium coccoides* group, *Clostridium leptum* subgroup, *Bacteroides-Prevotella* group) (Tannock 2003; Eckburg, et al. 2005). Thus, bacteria dominate the bowel community. Fungi and Archaea may also be resident, but comprise less than 0.05 percent and 1 percent of the total inhabitants, respectively (Miller and Wolin 1983; Simon and Gorbach 1984). Much of this information has been generated through the application of nucleic acid-based methodologies, most of which target the nucleotide base sequence of small ribosomal subunit RNA (16S rRNA in the case of bacteria)

which provides a cornerstone of microbial taxonomy. An earlier estimate of the number of bacterial species that might be resident in the human large bowel was based on bacteriological culture. Four hundred species seemed a likely number by extrapolation from what had already been cultured (Finegold, et al. 1974; Moore and Holdeman 1974). Nucleic acid-based methods of detection suggest that about 50 percent of the bacterial cells seen microscopically in feces cannot yet be cultured in the laboratory, even when accounting for the fact that some of the bacteria are dead (Tannock, et al. 2000; Ben-Amor, et al. 2005). This phenomenon manifested even more dramatically in terrestrial and aquatic ecosystems was, based on traditional bacteriological experience, totally unexpected and has been called “the great plate count anomaly” (Amann, et al. 1995). “Operational taxonomic units” (OTU; molecular species) never encountered in culture-based bacteriology are detectable by the molecular methods. Estimates of biodiversity now seem to continually inflate, probably because many of the 16S rRNA gene sequences in databanks differ by one nucleotide base. These are likely to be sequences representing the same OTU but containing sequencing errors. Curiously, therefore, we do not really accurately know what the bowel community is composed of.

Probiotics are products that contain living microbial cells, usually of genera that produce major amounts of lactic acid as fermentation product, that are consumed with the aim of benefiting the consumer’s health. Traditionally, it has been assumed that consumption of the probiotic bacteria will result in a modification of the proportions of bacterial species comprising the resident bacterial community of the large bowel of humans. This has been tested by the application of nucleic acid-based methods, of general use in microbial ecology, that enable specific bacteria (species and even strains) to be tracked during the course of probiotic studies (see Table 9.1 for examples). As alluded to above, the composition of the gut microflora can only be evaluated comprehensively in these studies by using nucleic acid-based methods. There are two main reasons why this is so:

1. The majority of members of the gut bacterial community of humans cannot yet be cultivated in the laboratory by traditional bacteriological methods. The starting point for nucleic acid-based methods is the extraction of bacterial DNA or RNA directly from the fecal or other sample of interest. 16S rRNA or the gene that encodes it has become a cornerstone of bacterial classification because it contains regions of nucleotide base sequence that are highly conserved across the bacterial world and that are interspersed with hypervariable regions (V regions). These hypervariable regions contain the “signatures” of phylogenetic groups and even species (Woese 1987). For this reason, hypervariable regions of 16S rRNA (or 16S rRNA gene sequences) are the basis of the analytical methods. Bacterial DNA or RNA extracted from the samples (in theory nucleic acid from all the bacterial types in the sample will be represented in the extracts) and polymerase chain reaction (PCR) amplification (reverse transcription-PCR in the case of RNA extracts) of the 16S rRNA gene in part or complete, is carried out. Clone libraries of the 16S rRNA genes can be made, and the clones sequenced, thus producing a kind of catalog of the bacterial constituents of the

Table 9.1 Nucleic Acid-based Analytical Methods

Technique	Description	Example
Colony Hybridization	Colonies are transferred to a nylon membrane, lysed and hybridized with labeled oligonucleotide probes (radioactive, enzymatic, fluorescent); probes will bind to specific target sequences in bacterial nucleic acids (RNA, genome)	Kaneko and Kurihara 1996
Dot-Blot Northern/ Southern Blotting	Hybridization of RNA or DNA with specific labeled probes; nucleic acids are immobilized in a dot or in a band after an electrophoretic run	Kaneko and Kurihara 1996
Fluorescence <i>In Situ</i> Hybridization (FISH)	Hybridization with fluorescent labeled probes of cells fixed on slides with wells, or in tubes	Franks, et al. 1998
Flow Cytometry	Fluorescently tagged bacterial cells are enumerated by flow cytometry	Vaughan et, al. 2000
Plasmid profiling	Pattern of plasmids in agarose gel	Tannock, et al. 1990
Restriction Fragment Length Polymorphism (RFLP)	Pattern of DNA fragments generated by enzymatic digestion and agarose gel electrophoresis	Kullen, et al. 1997
Terminal restriction fragment length polymorphism (T-RFLP)	Fluorescently tagged amplicons of specific sequences are produced during PCR by using labeled primers; subsequent restriction digests and visualization of the fluorescent-labeled terminal fragments on high-resolution sequencing gels produces the T-RFLP patterns	Kaplan, et al. 2001
Pulsed Field Gel Electrophoresis (PFGE)	RFLP of very large fragments of the whole genome generated using rare-cutting restriction enzymes, and separated using a pulsed field	McCartney, et al. 1996
Ribotyping	Modification of RFLP in which bacterial DNA is digested, and the fragments are separated by agarose electrophoresis, transferred to a membrane and hybridized with a probe targeting 5S, 16S or 23S rRNA genes	McCartney, et al. 1996
Amplified rDNA Restriction Analysis (ARDRA)	Restriction digests of 16S rRNA genes generate profiles of DNA fragments	Ventura, et al. 2000
Randomly Amplified polymorphic DNA (RAPD)	Short arbitrary primers and low stringency used to randomly amplify DNA fragments, which are separated by agarose electrophoresis to give a fingerprint	O'Sullivan and Kullen 1998

(continued)

Table 9.1 Nucleic Acid-based Analytical Methods (continued)

Technique	Description	Example
Specific PCR	Group- or species-specific PCR to detect certain groups of bacteria (qualitative)	Wang, et al. 1996
Multiplex-PCR	Simultaneous PCR amplification of a number of target DNA regions using more than one set of specific primers in the reaction mix	O'Sullivan 1999
Arbitrary Primed PCR (AP-PCR)	Only one short primer (10 to 12 bases) of arbitrary sequence anneals to the template DNA under low stringent conditions to a region where it exhibits nearest homology	O'Sullivan 1999
Triplet Arbitrary Primed PCR (TAP-PCR)	Introduction of specific changes (e.g., annealing temperature) to three otherwise identical reactions run in parallel to identify amplicons susceptible to changes. Primers are targeted to conserved regions.	O'Sullivan 1999
Denaturing Gradient Gel Electrophoresis (DGGE)	Separation of DNA fragments based on differences in chemical stability, through a linearly increasing gradient of denaturants (urea, formamide)	Walter, et al. 2001
Temperature Gradient Gel Electrophoresis/ Temporal Temperature Gel Electrophoresis (TGGE/TTGE)	Same as above but using temperature as denaturing factor	Zoetendal, et al. 1998
Cloning library	PCR amplification of 16S rRNA genes from a sample, and subsequent cloning of the amplicon in a vector to generate a bank of individual 16S rRNA gene clones ready for sequence and phylogenetic analysis	Wilson and Blitchington 1996
Real-time quantitative PCR	A reporter probe labeled with a reporter dye and a quencher molecule is used to measure the real-time accumulation of specific PCR product	Requena, et al. 2002

ecosystem (Suau, et al. 1999). From this sequence information, it is possible to derive DNA probes that can be labeled with fluorescent dyes and that specifically target variable regions of the 16S rRNA. This enables enumeration of the various phylogenetic groups of bacteria inhabiting the human gut, regardless of whether they can be cultured or not (Franks, et al. 1998; Sghir, et al. 2000). Deriving a library of hundreds of clones for every sample that needs to be investigated is logistically impossible. Even analysis using DNA probes is a serious undertaking and requires a semi-automated system such as fluorescence-

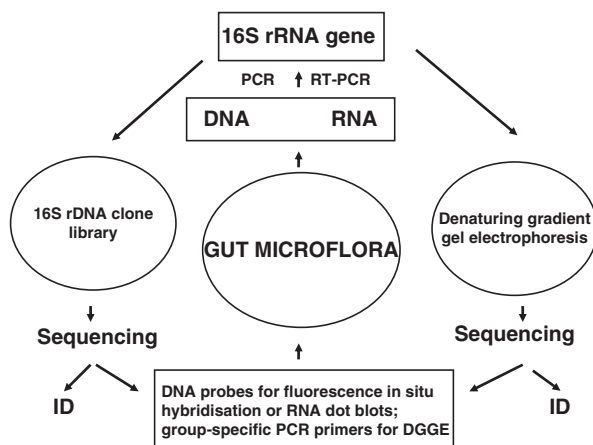


Fig. 9.1 Flow chart showing the inter-relatedness of nucleic acid-based methods that can be used to analyze complex bacterial communities. ID: bacterial identification; DGGE: denaturing gradient gel electrophoresis; PCR: polymerase chain reaction; RT-PCR: reverse transcription-PCR

activated-flow-cytometry for unbiased results to be obtained. A relatively simple, semi-quantitative screening method to compare the bacterial composition of multiple samples is provided by PCR combined with denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis (Zoetendal, et al. 1998) (Figure 9.1).

2. Detection of specific bacterial strains requires the use of genetic fingerprinting methods. These methods usually involve the generation of DNA fragment polymorphic profiles in agarose gels as the result of fingerprinting DNA extracted from pure cultures of bacteria. A number of fingerprinting methods have been described. In general, these methods rely on generating DNA fingerprints by restriction endonuclease digestion, or by PCR (Table 9.1).

2 Tried and Tested Methods in Probiotic Studies

In practice, four nucleic acid-based methods have been shown to be effective in the analysis of bacterial communities of the gut in relation to probiotic consumption: PCR coupled with denaturing gradient gel electrophoresis (PCR/DGGE/TTGE), fluorescence *in situ* hybridization (FISH), terminal restriction fragment length polymorphism (T-RFLP), and genetic fingerprinting by pulsed field gel electrophoresis (PFGE) of DNA digests to distinguish between bacterial strains (Figs. 9.2 through 9.5). It should be noted that in all of the methods that do not require bacterial culture, nucleic acids originating in both dead or living bacterial cells are detected.

2.1 PCR/DGGE/TTGE

DNA or RNA is extracted directly from intestinal or fecal samples. Then hypervariable 16S rRNA gene sequences are amplified using PCR primers that anneal with conserved sequences that span the selected V regions. One of the PCR primers has a GC-rich 5 end (GC clamp) to prevent complete denaturation of the DNA fragments during gradient gel electrophoresis. To separate the 16S fragments amplified from different types of bacteria and present in the PCR product, a polyacrylamide gel is used. In DGGE, the double-stranded 16S fragments migrate through a polyacrylamide gel containing a gradient of urea and formamide until they are

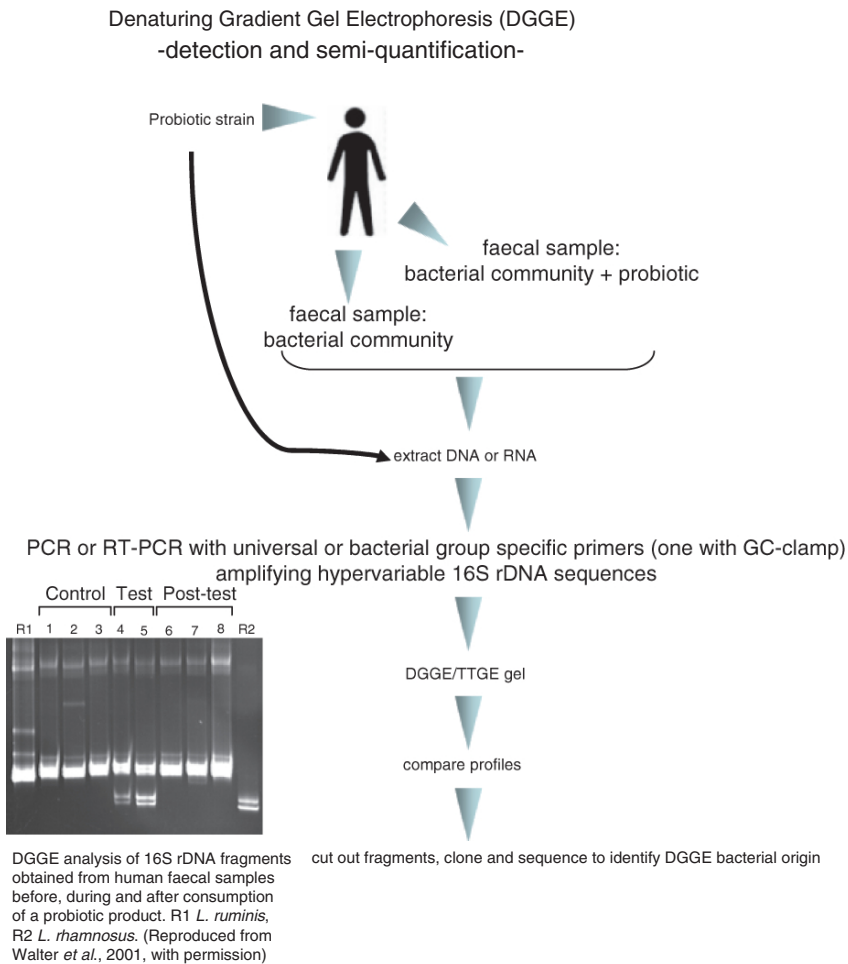


Fig. 9.2 Overview of the use of denaturing gradient gel electrophoresis methodology in probiotic studies

partially denatured by the chemical conditions. The fragments do not completely denature because of the GC clamp, and migration is radically slowed when partial denaturation occurs. Because of the variation in the 16S sequences of different bacterial species, chemical stability is also different; therefore different 16S 'species' can be differentiated by this electrophoretic method. Similarly, in TTGE, the 16S sequences can be separated by gradually increasing the temperature of the polyacrylamide gel during electrophoresis. Separation is achieved on the basis of differing temperature stability of the 16S fragments. These methods generate a profile of the numerically predominant members of the bacterial community (Muyzer and Smalla 1998). Individual DNA fragments can be cut from DGGE/TTGE gels, further amplified and cloned, then sequenced. The sequence can be compared to those in gene databanks to obtain identification of the bacterium from which the 16S sequence originated. Depending on the length of the sequence, identification to at least bacterial phylum can be made (Zoetendal, et al. 1998). In a further development of this methodology, PCR primers specific for bacterial groups can be derived. These primers generate a profile of the species comprising, for example, a specific bacterial genus within the bacterial community (Walter, et al. 2001; Knarreborg, et al. 2002; Requena, et al. 2002) (Fig. 9.2). Advantages of the PCR/DGGE/TTGE technology include:

1. No need to know which bacteria are present in the community at the outset.
2. Community profiles (snapshots of community composition) of many samples can be compared in one gel, or between gels if a standard profile is included in each gel.
3. Bacterial diversity (richness) can be compared between samples using DNA as template (taxonomic analysis), as well as bacterial activity (metabolic; the ribosome per cell ratio is roughly proportional to growth rate of the bacteria) using RNA as template.
4. Samples can be screened for changes in the composition of the community (appearance, disappearance of fragments, changes in intensity of staining of fragments).
5. Affected populations can be identified by sequencing DNA fragments.
6. Identification of bacteria by sequence can enable oligonucleotide probes to be derived for quantitative analysis.
7. Culture of bacteria is not required.
8. Samples can be frozen for storage and transportation to the analytical laboratory.

The disadvantages of PCR/DGGE/TTGE include:

1. At best, semi-quantitative analysis is obtained.
2. PCR can generate inaccuracies such as chimeric DNA sequences or by preferential amplification of some sequences over others (PCR bias).
3. The numerically dominant populations in the community are represented in the profiles. Bacteria that form less than 1 percent of the total community are not detected in the profiles when universal bacterial PCR primers are used; this limitation can be overcome by the use of group-specific primers.

4. DNA fragments of different nucleotide base sequence may co-migrate.
5. Heterogeneity of *rrn* operons within a single cell means that a bacterial species can be represented by more than one fragment in the profile.

An example of the use of PCR/DGGE/TTGE in a probiotic study is provided by the work of Satokari and colleagues (2001). A group of human subjects were administered *Bifidobacterium lactis* (about 3×10^{10} cfu per day for two weeks). PCR/DGGE analysis of the fecal microbiota of the subjects detected the presence of *B. lactis* during the period of probiotic administration, but not once consumption of the bacteria had ceased.

2.2 FISH

DNA (oligonucleotide) probes target specific rRNA sequences (16S or 23S) within ribosomes to which they hybridize. The probes are 5' labeled feet long, labeled with a fluorescent dye which permits both detection and quantification of specific bacterial populations (Fig. 9.3). Bacterial cells within which hybridization with a probe has occurred fluoresce and hence can be detected and counted by epifluorescence microscopy (preferably automated) or fluorescence-activated flow cytometry. The following technical aspects of FISH should not be overlooked:

1. Cell wall permeability. The physiological state of the bacterial cell may influence the permeability of the cell to DNA probes. Therefore, a permeabilization step is needed to standardize intracellular access of DNA probes to their targets (Lay, et al. 2005).
2. *In situ* accessibility. Within the cell the secondary structure of rRNA molecules and their molecular interactions within the ribosome may hinder the access of the probes to their target sites. This *in situ* accessibility influences the amount of fluorescence generated from the probe (Fuchs, et al. 1998). A high degree of *in situ* accessibility facilitates the binding of the probe to its target site and, therefore, results in the emission of a bright fluorescent signal. The determination of the brightness of fluorescence (probe relative fluorescence) conferred by a probe is a means of evaluating its *in situ* accessibility (Fuchs, et al. 1998; Lay, et al. 2005). Modeling of the secondary structure of 16S rRNA molecules allows *in silico* investigation of the *in situ* accessibility of the entire molecule (Fuchs, et al. 1998; Kumar, et al. 2005; Saunier, et al. 2005) and the target site can be assessed in terms of accessibility. If the target region is located in a poorly- or non-accessible site, helper probes (unlabeled oligonucleotides) that are complementary to regions adjacent to the probe's target site can be derived, promoting the binding of the probe and, therefore, amplifying the fluorescence signal (Fuchs, et al. 2000; Saunier, et al. 2005; Dinoto, et al. 2006).
3. Ribosomal content/metabolic activity of the cell. The greater the number of ribosomes per bacterial cell, the higher their metabolic activity. Therefore, bacterial cells in a quiescent state have weak fluorescence and may escape detection.

Fluorescence *In Situ* Hybridization (FISH)

-detection and quantification-

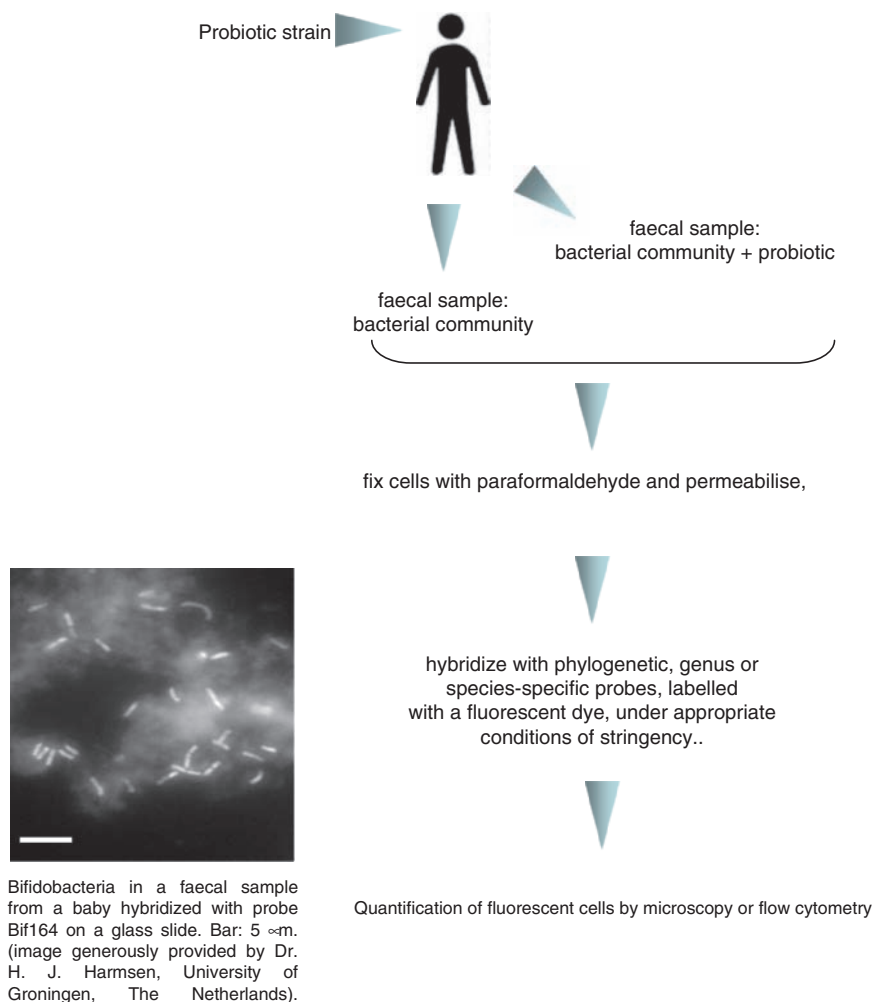


Fig. 9.3 Overview of the use of fluorescence *in situ* hybridization methodology in probiotic studies

4. Hybridization conditions. The stringency of hybridization depends on three parameters: temperature, salt concentration, and formamide concentration of the hybridization solution. The manipulation of these factors influences the specificity of hybridization and, hence, detection and quantification.

5. Increasing database biodiversity. Cross-hybridization with non-target cells and partial coverage of a bacterial phylogenetic group are the main criteria for excluding probes from use. More than 400,000 16S rRNA sequences are stored in the Ribosomal Database (<http://rdp.cme.msu.edu>), permitting the *in silico* design and validation of probes that will target different phylotypes resident in the human gut. Several of the current probes were designed using older versions of the Ribosomal Database, so continual reassessment of specificity and coverage of these probes is essential to update and confirm their continuing reliability.
6. Epifluorescence microscopic detection. Laborious and time-consuming, manual microscopic enumeration requires careful attention by the operator. A lower detection limit of about 10^6 bacteria per gram of feces can be achieved (Welling, et al. 1997). An automated method of counting fluorescent bacterial cells has been developed by coupling fluorescence microscopy to a computerized system of image analysis (Jansen, et al. 1999). Using this automated counting device, the lower detection threshold has been estimated to be 10^7 bacteria per gram of feces. Therefore, only the more numerous members of the bacterial community can be detected. Nevertheless, identification of individual bacterial cells, as well as morphological and topographical information, are valuable characteristics of fluorescence microscopy.
7. Fluorescence-activated flow cytometry detection. Combined with flow cytometry, FISH provides a high throughput quantitative and qualitative method of analysis. Rapid and easy to set up, flow cytometry combines quantitative and multiparametric analysis (size, internal granularity, fluorescence signal). A lower threshold of detection of 0.4 percent relative to the total number of bacteria determined with the universal bacterial probe EUB338 has been demonstrated (Lay 2004).

FISH has provided important data concerning the composition of the bacterial community of the gut in probiotic studies as demonstrated by the following examples.

- (a) The composition of the fecal bacterial community of 10 healthy subjects was monitored before (six-month control period), during (six-month test period) and after (three-month post-test period) the administration of a probiotic product containing *Lactobacillus rhamnosus* DR20 (daily dose, 1.6×10^9 lactobacilli). FISH, combined with automated microscopy, showed that long-term consumption of *Lactobacillus rhamnosus* DR20 did not affect the proportions of the dominant bacterial populations of feces: *Bacteroides*, *Clostridium coccoides-Eubacterium rectale*, *Atopobium*, *Bifidobacterium* and the gram positive, low-G+C content group 2 bacteria (Tannock, et al. 2000).
- (b) The establishment of the fecal community of breastfed infants at risk of allergic diseases (n=132) was monitored throughout the first two years of life. Infants received either *Lactobacillus rhamnosus* GG (daily dose, 1.0×10^{10} lactobacilli) or a placebo throughout the first six months of life. The fecal bacteria were analyzed using FISH combined with fluorescence microscopy at six, 12, 18 and 24 months after birth. The administration of the probiotic did not alter

the normal establishment of the gut community *Bifidobacterium*, *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella* and the *Clostridium histolyticum* groups (Rinne, et al. 2006).

- (c) The effects of administering raffinose and encapsulated *Bifidobacterium breve* JCM 1192^T on the rat cecal community has been investigated (Dinoto, et al. 2006). Twenty-four rats were divided into four groups and fed different diets for three weeks: basal diet (group BD), basal diet supplemented with raffinose (group RAF), basal diet supplemented with encapsulated *Bifidobacterium breve* JCM 1192^T (group CB), and basal diet supplemented with both raffinose and *Bifidobacterium breve* JCM 1192^T (group RCB). The combination of raffinose and *Bifidobacterium breve* JCM 1192^T was referred to as a synbiotic (prebiotic plus probiotic). A *Bifidobacterium breve*-specific oligonucleotide probe (PBR2), along with helper probes, was derived to monitor the influence of the diets on the composition of the cecal community. The probiotic strain was only detected in samples collected from the RCB group (7.3 percent of the total bacterial population). This result indicated that the addition of raffinose to the diet enabled *Bifidobacterium breve* JCM 1192^T to proliferate in the cecum.
- (d) The consumption of a fermented milk product containing *Lactobacillus casei* DN-114001 (daily dose of 3.0×10^{10} CFU) on the fecal bacterial community was monitored in 12 healthy subjects before (control period of one week), during (10 days supplementation) and after (10 days post-ingestion) probiotic administration (Rochet, et al. 2006). Probiotic supplementation did not alter the proportions of the dominant populations within the fecal community (*Eubacterium rectale-Clostridium coccoides*, *Faecalicobacterium prausnitzii*, *Bacteroides-Prevotella*, *Bifidobacterium*, *Atopobium*, *Lactobacilli-Enterococci* and Enterobacteriaceae).

The advantages of FISH are:

1. Culture of bacteria is not required.
2. Fixed samples can be stored and transported to the analytical laboratory.
3. Detection and quantification of specific bacterial types can be achieved.
4. PCR is not required.
5. Identification of individual cells, morphological descriptions and observations on the distribution of bacterial cells can be made, including in histological sections of tissue.
6. High-throughput analysis can be achieved in combination with flow cytometry.

Disadvantages include:

1. Target sequences for bacteria in the sample must be known in advance.
2. Numerically predominant bacterial species can be detected and enumerated, but minor components of the bacterial community will be missed.
3. Some gram positive bacteria may be missed because they are not adequately permeabilized.
4. Probe specificity can be guaranteed only for known bacteria.
5. Automated analysis is required for high-throughput and accurate enumerations.

2.3 T-RFLP

Terminal restriction fragment profiles are generated and analyzed in a series of steps that combine PCR, restriction endonuclease digestion and gel electrophoresis. The method is based on the premise that each species in a bacterial community will produce a characteristic DNA fingerprint due to restriction fragment length polymorphism (RFLP) of the selected sequence. The complexity of the RFLP community fingerprint is simplified, however, by the use of a PCR primer that has a terminal fluorescent tag (Fig. 9.4). The method is intended to measure both the diversity (richness) and evenness (population sizes) of the community. DNA is extracted from a sample containing a bacterial community and is subjected to PCR using primers that anneal to conserved regions of the target gene (most often the 16S rRNA gene). One PCR primer is labeled, often at the 5'-end, with a fluorescent molecule. The amplified DNA sequences are then digested with a restriction endonuclease that recognizes a four-base sequence. The digested amplicons are subjected to electrophoresis in either a polyacrylamide gel or a capillary gel apparatus; usually a DNA sequencer with a fluorescence detector. By this means, the DNA fragments are separated and only the 5'-labeled restriction fragments are detected. Automated fragment analysis programs are used to calculate the size (length) of the fragment by comparing its migration distance (peak retention time) to that of a DNA size standard. The computer programs integrate the electropherograms and measure peak area (quantification of specific population). The T-RFLP patterns can then be compared between samples, and specific peaks can be associated with the bacteria of origin by comparison to a clone library or by predictions made from an existing database of sequences (Kitts 2001).

The advantages of the method include:

1. Bacteriological culture is not required.
2. Both richness and evenness of the community can be measured.

Disadvantages include:

1. A high degree of reproducibility of restriction endonuclease digestions is required.
2. A DNA sequencer scanner is required.
3. PCR can generate inaccuracies such as chimeric DNA sequences or preferential amplification of some sequences over others.
4. Diversity might be underestimated because terminal restriction size could be the same for all members of a bacterial phylogenetic division (conservation of restriction endonuclease recognition sites).

An example of the use of T-RFLP in a probiotic study is provided by the work of Kaplan and colleagues (2001) in which rat fecal samples were analyzed to follow the fate of *Lactobacillus acidophilus* NCFM that had been fed to the animals. A large T-RFLP peak was detected in samples from rats fed the *Lactobacillus* strain, whereas only a small peak was present in the corresponding position of the electropherogram of untreated rats (Fig. 9.4).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) -detection and quantification-

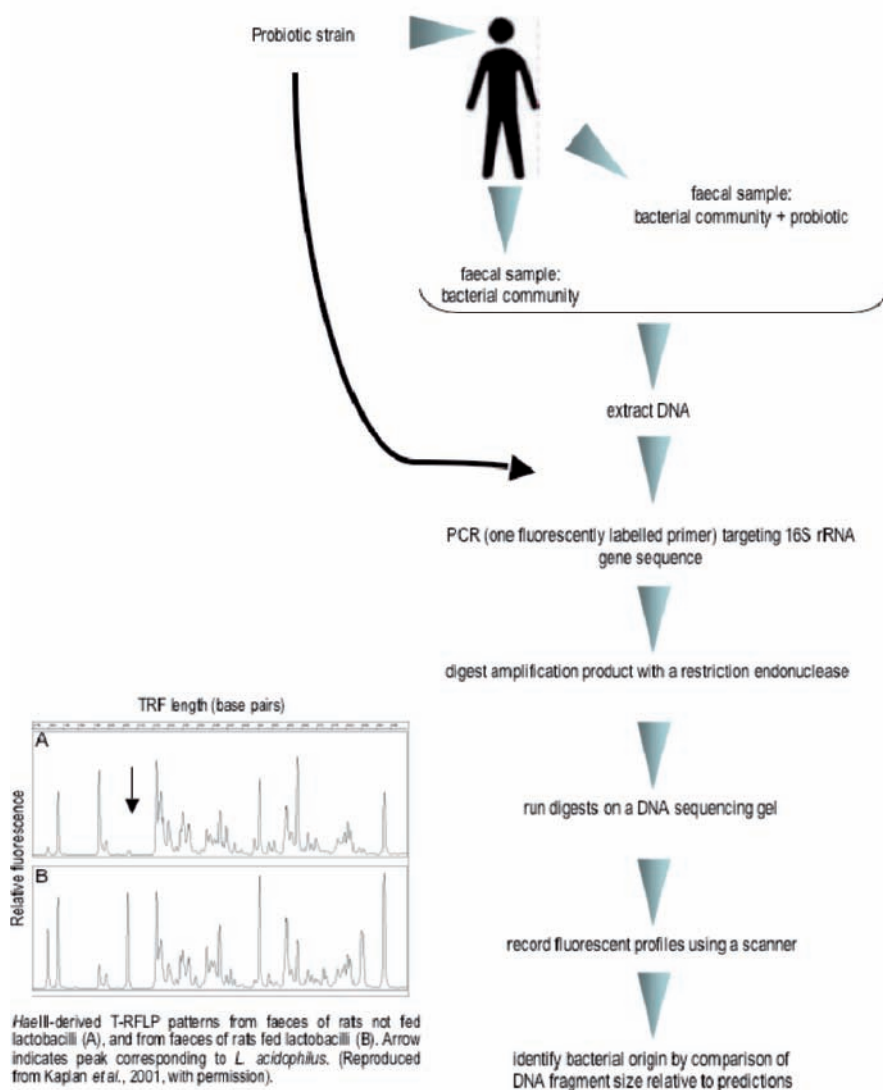


Fig. 9.4 Overview of the use of terminal restriction fragment length polymorphism methodology in probiotic studies

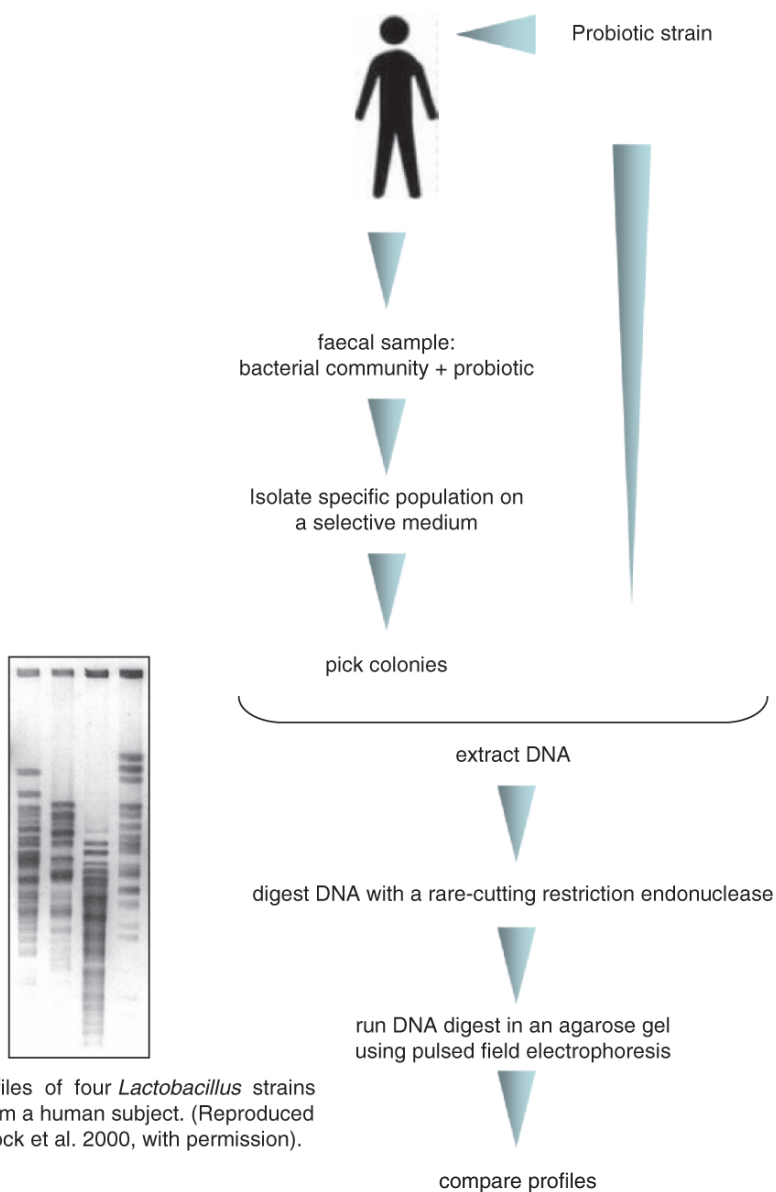
2.4 PFGE

Chromosomal DNA is extracted from pure cultures of bacteria. The DNA is digested by a restriction endonuclease chosen on the basis of the mol% G+C content of the DNA of the bacterial species, and on the recognition sequence of the enzyme. An endonuclease that will cut the DNA rarely is desired so that a relatively small number of DNA fragments result from the digestion and a relatively simple pattern will be generated in the electrophoretic gel. The digestion generates large fragments of DNA that would not separate by the usual agarose gel electrophoresis that is based on molecular sieving. Therefore, pulsed field gel electrophoresis is used in which the mixture of fragments in the DNA digest are exposed to alternating electrical fields that force the fragments to change orientation, rather than migrate through the agarose gel immediately after the electrical field is changed from one direction to another. The rate of re-orientation is size dependent, so larger molecules change direction more slowly than smaller ones. The pulse time (the time spent in a field of particular direction) is varied and this dictates the DNA class size that spends most of the time re-orientating rather than migrating. The DNA fragments are thus separated by the retardation of net movement rather than by sieving. The pattern of fragments generated in the gel represents the genetic fingerprint of the bacterial culture and is characteristic of that strain of bacteria (Gardiner 1991) (Fig. 9.5). PFGE of DNA digests provides an ideal method by which a probiotic bacterial strain can be tracked during the course of a probiotic study. The bacterial group of interest can be selectively cultured and colonies are randomly picked to obtain pure cultures. The genetic fingerprint of these isolates is then determined by PFGE of DNA digests, and compared with that of the probiotic bacterial strain. The presence or absence of the probiotic strain in fecal samples can be determined by this method. Additionally, the proportion that the probiotic strain comprises of the total bacterial population can be estimated. The advantage of PFGE of DNA digests is that a specific strain can be tracked during studies aimed at determining the persistence of the probiotic strain in the gut, and its impact on the composition of a specific bacterial population. Disadvantages include the requirement for bacteriological culture and the immense logistical effort required to genetically fingerprint hundreds or thousands of bacterial isolates.

An example of the use of genetic fingerprinting in a probiotic study is provided by the work of Tannock and colleagues (2000) who analyzed the composition of the *Lactobacillus* populations present in the feces. The composition of the fecal bacterial community of 10 human subjects was monitored before (control period of six months), during (test period of six months) and after (post-test period of three months) the administration of a milk product containing *Lb. rhamnosus* DR20 (daily dose of 1.6×10^9 lactobacilli). The composition of the *Lactobacillus* population of each subject was analyzed by PFGE for bacterial DNA digests to differentiate between DR20 and other strains present in the fecal samples. Consumption of the probiotic transiently altered the composition of the *Lactobacillus* populations of the subjects, but to varying degrees. The detection of DR20 among the numerically predominant strains was related to the presence or absence of a stable autochthonous

Pulsed Field Gel Electrophoresis of DNA digests (PFGE)

-differentiation of strains -

**Fig. 9.5** Overview of the use of pulsed field gel electrophoresis of DNA in probiotic studies

population of lactobacilli during the control period. The probiotic strain did not predominate in samples collected from subjects with *Lactobacillus* populations of stable composition.

3 Conclusion

The most important outcome of using nucleic acid-based methods in probiotic studies has been to reveal the remarkable individuality and homeostasis of the large bowel (fecal) communities of humans. The genetic fingerprint of the community remains the same in samples collected during long-term studies, even 15 months in duration (Tannock, et al. 2000). Homeostasis of microbial communities is a common feature of ecological studies in which a steady state is generated by the organisms themselves. Competition for nutrients and space, the inhibition of one group by the metabolic products of another group, and predation and parasitism all contribute to the regulation of populations in particular proportions, one to the other. Because all of the ecological niches are filled in a regulated microbial community, it is extremely difficult for "alien" microbes, accidentally or intentionally introduced into an ecosystem, to establish (Alexander 1971). They have no way of earning their living in the ecosystem since all possible niches have been filled. Competitive exclusion ("niche exclusion") is a general ecological phenomenon and pathogens are not the only aliens to enter the gut ecosystem. It applies equally well to the introduction of food-associated bacteria and probiotic bacteria into the gut. These, too, are alien bacteria and they have only a transient existence in the ecosystem. Thus, in several studies in which a probiotic product was administered to human subjects, the probiotic strain was only detected in the feces while the probiotic continued to be consumed (Alander, et al. 1999; Dunne, et al. 1999; Tannock, et al. 2000; Satokari, et al. 2001; Spanhaak, et al. 2001). Once consumption of the probiotic product ceased, so too did excretion of the bacteria in the feces. Moreover, in the study reported by Tannock, et al. (2000), numbers of the probiotic strain were relatively low (10^5 to 10^6 per gram of feces) and were detected only irregularly in samples collected from about 40 percent of the subjects who had a pre-existing, stable *Lactobacillus* population resident in their gut. The remainder of the subjects did not have stable *Lactobacillus* populations and the probiotic strain could be detected in all of their fecal samples during the period of probiotic use (Tannock, et al. 2000). Therefore, the outcome of probiotic consumption for consumers is unpredictable at the outset of studies: in some subjects the probiotic will be easily detectable in the feces; in others it will be irregular. The impact of probiotic consumption on humans may, therefore, vary according to the pre-existing composition of the intestinal bacterial community and the degree of competitive exclusion that it generates. There are, doubtless, many mechanisms that operate in the gut ecosystem that are important in competitive exclusion. A collection of bacterial species that suppressed the growth of *Escherichia coli* in the gut of mice fed a refined diet, for example, did not have the same effect when a different diet was fed. When a crude diet was fed to the animals, a larger collection of bacteria was required to

produce the inhibitory effect (Freter 1988). In general, therefore, there is a lack of evidence to support the traditional view that probiotic administration alters the composition of the resident bacterial community.

Largely for historical reasons, lactobacilli and bifidobacteria are predominant in the use of microbes as probiotics (Goldin and Gorbach 1992). Another important observation about the application of nucleic acid-based analytical methods is that neither of these groups is a major component of an adult human's fecal community. Lactobacilli are present at most at 10^8 per gram (wet weight) of feces (0.1 percent of the total community), but usually at about 10^6 per gram (0.001 percent) when detectable (Tannock, et al. 2000). According to American observations, 25 percent of humans do not have lactobacilli at detectable levels in their feces (single sample, Finegold, et al. 1983). Recent studies indicate that most of the lactobacilli detected in human feces are transient in the gut because they are detected intermittently in fecal samples from subjects in temporal studies (Tannock, et al. 2000; Reuter 2001). These lactobacilli belong to species that are commonly associated with foods such as cheese, fermented meats and vegetables, and have probably been introduced into the gut with the food (Walter, et al. 2001). Some may be members of the oral microflora and originate in the saliva. Only one species has been observed to attain moderate-sized populations that persist as long-term inhabitants of the human gut: *Lactobacillus ruminis* (Tannock, et al. 2000; Reuter 2001). Bifidobacteria are more numerous than lactobacilli and are harbored by most adult humans, but on average comprise only a few percent of the total bacterial community (Langendijk, et al. 1995; Sghir, et al. 1995; Franks, et al. 1998). They are, however, the predominant bacteria in the gut of children during the first month of life (Harmsen, et al. 2000).

It is also clear that a combination of methods is needed to offer optimal analysis of the human gut bacterial community. Ideally, both culture-dependent and culture-independent methods should be used, and qualitative and quantitative data should be recorded. The logistics of gut community analysis in probiotic studies are formidable, but the following protocol is feasible and has been used in a human study (Tannock, et al. 2000; Walter, et al. 2001).

1. Qualitative screening of the bacterial community in samples using PCR/DGGE/TTGE and universal bacterial primers.

Table 9.2 Useful Culture Media for Probiotic Studies¹

Medium	Purpose
Supplemented brucella blood agar	Total aerobic and anaerobic counts
Bacteroides bile esculin agar	<i>Bacteroides fragilis</i> group
Egg yolk agar	Lecithinase-producing clostridia
Rogosa SL agar	Lactobacilli and bifidobacteria
MacConkey agar	Enterobacteriaceae
Bile esculin agar	Enterococci
Sabouraud dextrose agar	Yeasts

¹See Summanen, et al. 1993 and Tannock, et al. 2000 for further details and other examples of bacteriological culture media.

2. Qualitative detection of the probiotic species using PCR/DGGE/TTGE and group-specific primers.
3. Quantification of major phylogenetic bacterial groups using FISH and of selected bacterial populations by culture (Table 9.2).
4. Quantification of the probiotic population by selective culture and detection, and semi-quantification of the probiotic strain by PFGE of DNA digests.

A combination of methods like these provides a comprehensive view of the impact of probiotic consumption on the large bowel community and could be used as a paradigm for probiotic studies concerning human subjects.

References

- Alander, M., Satokari, R., Korpela, R., Saxelin, M., Vilpponen-Salmela, T., Mattila-Sandholm, T., von Wright, A. 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. *Appl. Environ. Microbiol.* 65, 351–354.
- Alexander, M. 1971. *Microbial ecology*. John Wiley and Sons, New York.
- Amann, R. I., Ludwig, W., Schleifer, K.-H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169.
- Ben-Amor, K., Heilig, H., Smidt, H., Vaughan, E. E., Abee, T., de Vos, W. M. 2005. Genetic diversity of viable, injured, and dead fecal bacteria assessed by fluorescence-activated cell sorting and 16S rRNA gene analysis. *Appl. Environ. Microbiol.* 71, 4679–4689.
- Dunne, C., Murphy, L., Flynn, S., O'Mahony, L., O'Halloran, S., Feeney, M., Morrissey, D., Thornton, G., Fitzgerald, G., Daly, C., Kiely, B., Quigley, E. M., O'Sullivan, G. C., Shanahan, F., Collins, J. K. 1999. Probiotics: from myth to reality. Demonstration of functionality in animal models of disease and in human clinical trials. *Antonie van Leeuwenhoek* 76, 279–292.
- Dinoto, A., Suksomcheep, A., Ishizuka, S., Kimura, H., Hanada, S., Kamagata, Y., Asano, K., Tomita, F., Yokota, A. 2006. Modulation of rat cecal microbiota by administration of raffinose and encapsulated *Bifidobacterium breve*. *Appl. Environ. Microbiol.* 72, 784–792.
- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., Relman, D. A. 2005. Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638.
- Finegold, S. M., Attebury, R., Sutter, V. L. 1974. Effect of diet on human fecal flora: comparison of Japanese and American diets. *Am. J. Clin. Nutr.* 27, 1456–1469.
- Finegold, S. M., Sutter, V. L., Mathisen, G. E. 1983. Normal indigenous intestinal flora. In: Hentges, D. J. (Ed), *Human intestinal flora in health and disease*. Academic Press, New York, pp. 3–31.
- Freter, R. 1988. Mechanisms of bacterial colonization of the mucosal surfaces of the gut. In: Roth, J. A. (Ed), *Virulence mechanisms of bacterial pathogens*. American Society for Microbiology, Washington DC, pp. 45–60.
- Franks, A., Harmsen, H. J. M., Raangs, G. C., Jansen, G. J., Schut, F., Welling, G. W. 1998. Variations of bacterial population in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* 64, 3336–3345.
- Fuchs, B. M., Glockner, F. O., Wulf, J., Amann, R. 2000. Unlabeled helper oligonucleotides increase the *in situ* accessibility to 16S rRNA of fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* 66, 3603–3607.
- Fuchs, B. M., Wallner, G., Beisker, W., Schwiippl, I., Ludwig, W., Amann, R. 1998. Flow cytometric analysis of the *in situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. *Appl. Environ. Microbiol.* 64, 4973–4982.
- Gardiner, K. 1991. Pulsed field gel electrophoresis. *Analytical Chemistry* 63, 658–665.

- Goldin, B. R., Gorbach SL. 1992. Probiotics for humans. In: Fuller, R. (Ed), Probiotics. The scientific basis. Chapman and Hall, London, pp. 355–376.
- Harmsen, H. J. M., Raangs, G. C., He, T., Degener, J. E., Welling, G. W. 2002. Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. Appl. Environ. Microbiol. 68, 2982–2990.
- Jansen, G. J., Wildeboer-Veloo, A. C., Tonk, R. H., Franks, A. H., and Welling, G. W. 1999. Development and validation of an automated, microscopy-based method for enumeration of groups of intestinal bacteria. J. Microbiol. Methods 37, 215–221.
- Kaneko, T., Kurihara, H. 1996. Digoxigenin-labeled deoxyribonucleic acid probes for the enumeration of bifidobacteria in fecal samples. J. Dairy Sci. 80, 1254–1259.
- Kaplan, C. W., Astaire, J. C., Sanders, M. E., Reddy, B. S., Kitts, C. L. 2001. 16S ribosomal DNA terminal restriction fragment pattern analysis of bacterial communities in feces of rats fed *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. 67, 1935–1939.
- Kitts, C. L. 2001. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. Curr. Issues Intest. Microbiol. 2, 17–25.
- Knarreborg, A., Simon, M. A., Engberg, R. M., Jensen, B. B., Tannock, G. W. 2002. Effects of dietary fat source and subtherapeutic levels of antibiotic on the bacterial community in the ileum of broiler chickens at various ages. Appl. Environ. Microbiol. 68, 5918–5924.
- Kullen, M. J., Amann, M. M., O'Shaughnessy, M. J., O'Sullivan, D. J., Busta, F. F., Brady, L. J. 1997. Differentiation of ingested and endogenous bifidobacteria by DNA fingerprinting demonstrates the survival of an unmodified strain in the gastrointestinal tract of humans. J. Nutr. 127, 89–94.
- Kumar, Y., Westram, R., Behrens, S., Fuchs, B., Glockner, F. O., Amann, R., Meier, H., Ludwig, W. 2005. Graphical representation of ribosomal RNA probe accessibility data using ARB software package. BMC Bioinformatics 6, 61.
- Langendijk, P., Schut, F., Jansen, G. J., Raangs, G. C., Kamphuis, G. R., Wilkinson, M. H. F., Welling, G. W. 1995. Quantitative fluorescence *in situ* hybridization of *Bifidobacterium* spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. 61, 3069–3075.
- Lay, C. 2004. Caractérisation moléculaire à haut débit de la diversité phylogénétique de la microflore digestive humaine. Ph.D. Thesis. Faculté de Pharmacie, Université Paris XI, Châtenay-Malabry, France.
- Lay, C., Sutren, M., Rochet, V., Saunier, K., Dore, J., Rigottier-Gois, L. 2005. Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human fecal microbiota. Environ. Microbiol. 7, 933–946.
- McCartney, A. L., Wenzhi, W., Tannock, G. W. 1996. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl. Environ. Microbiol. 62, 4608–4613.
- Muyzer, G., Smalla, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73, 127–141.
- Miller, T. L., Wolin, M. J. 1983. Stability of *Methanobacter smithii* populations in the microbial flora excreted from the human large bowel. Appl. Environ. Microbiol. 45, 317–318.
- Moore, W. E. C., Holdeman, L. V. 1974. Special problems associated with the isolation and identification of intestinal bacteria in fecal flora studies. Am. J. Clin. Nutr. 27, 1450–1455.
- O'Sullivan, D. J., Kullen, M. J. 1998. Tracking of probiotic bifidobacteria in the intestine. Int. Dairy J. 8, 513–525.
- O'Sullivan, D. J. 1999. Methods for analysis of the intestinal microflora. In: G. W. Tannock (Ed), Probiotics: A critical review. Horizon Scientific Press, Wymondham, UK, pp. 34–35.
- Requena, T., Burton, J., Matsuki, T., Munro, K., Simon, M. A., Tanaka, R., Watanabe, K., Tannock, G. W. 2002. Identification, detection, and enumeration of human *Bifidobacterium* species by PCR targeting the transaldolase gene. Appl. Environ. Microbiol. 68, 2420–2427.
- Reuter, G. 2001. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. Curr. Issues Intest. Microbiol. 2, 43–53.

- Rinne, M., Kalliomaki, M., Salminen, S., Isolauri, E. 2006. Probiotic intervention in the first months of life: short-term effects on gastrointestinal symptoms and long-term effects on gut microbiota. *J. Pediatr. Gastroenterol. Nutr.* 43, 200–205.
- Rochet, V., Rigottier-Gois, L., Sutren, M., Kremetscki, M. N., Andrieux, C., Furet, J. P., Tailliez, P., Levenez, F., Mogenet, A., Bresson, J. L., Meance, S., Cayuela, C., Leplingard, A., Dore, J. 2006. Effects of orally administered *Lactobacillus casei* DN-114 001 on the composition or activities of the dominant fecal microbiota in healthy humans. *Br. J. Nutr.* 95, 421–429.
- Saunier, K., Rouge, C., Lay, C., Rigottier-Gois, L., Dore, J. 2005. Enumeration of bacteria from the *Clostridium leptum* subgroup in human fecal microbiota using Clep1156 16S rRNA probe in combination with helper and competitor oligonucleotides. *Syst. Appl. Microbiol.* 28, 454–464.
- Satokari, R. M., Vaughan, E. E., Akkermans, A. D. L., Saarela, M., de Vos, W. M. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67, 504–513.
- Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P., Dore, J. 2000. Quantification of bacterial groups within the human fecal flora by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 66, 2263–2266.
- Simon, G. L., Gorbach, S. L. 1984. Intestinal flora in health and disease. *Gastroenterol.* 86, 174–193.
- Spanhaak, S., Havenaar, R., Schaafsma, G. 1998. The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *European J. Clin. Nutr.* 52, 899–907.
- Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G. R., Collins, M. D., Dore, J. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* 65, 4799–4807.
- Summanen, P., Baron, E. J., Citron, D. M., Strong, C., Wexler, H. M., Finegold, S. M. 1993. *Wadsworth Anaerobic Bacteriology Manual*. Star Publishing Company, Belmont, USA.
- Tannock, G. W. 2003. The intestinal microflora. In Fuller R, Perdigon G (Ed), *Gut flora. Nutrition, immunity and health*. Blackwell Press, Oxford, p. 1–23.
- Tannock, G. W., Fuller, R., Pedersen, K. 1990. *Lactobacillus* succession in the piglet intestinal tract demonstrated by plasmid profiling. *Appl. Environ. Microbiol.* 56, 1310–1306.
- Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J., Gopal, P. K. 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl. Environ. Microbiol.* 66, 2578–2588.
- Vaughan, E. E., Schut, F., Heilig, H. G. H. J., Zoetendal, E. G., de Vos, W., Akkermans, A. D. L. 2000. A molecular view of the intestinal ecosystem. *Curr. Issues Intest. Microbiol.* 1, 1–12.
- Ventura, M., Casas, I. A., Morelli, L., Callegari, M. L. 2000. Rapid amplified ribosomal DNA restriction analysis (ARDRA) identification of *Lactobacillus* spp. isolated from fecal and vaginal samples. *Syst. Appl. Microbiol.* 23, 504–509.
- Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., Hammes, W. P. 2001. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl. and Environ. Microbiol.* 67, 2578–2585.
- Wang, R. F., Cao, W. W., Cerniglia, C. E. 1996. PCR detection and quantification of predominant anaerobic bacteria in human and animal fecal samples. *Appl. Environ. Microbiol.* 62, 1242–1247.
- Welling, G. W., Elfferich, P., Raangs, G. C., Wildeboer-Veloo, A.C., Jansen, G. J., Degener, J. E. 1997. 16S ribosomal RNA-targeted oligonucleotide probes for monitoring of intestinal tract bacteria. *Scand. J. Gastroenterol.* 222, 17–19.
- Wilson, K. H., Blitchington, R. B. 1996. Human colonic biota studied by ribosomal DNA sequence analysis. *Appl. Environ. Microbiol.* 62, 2273–2278.
- Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* 51, 221–271.
- Zoetendal, E. G., Akkermans, A. D. L., de Vos, W. M. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* 64, 3854–3859.

Chapter 10

Bioinformatics for DNA Sequence-based Microbiota Analyses

Knut Rudi

Abstract Microbiota samples are often complex and difficult to define with respect to organism content. The emergence of growth-independent DNA-based techniques, however, has made data generation from microbiota samples relatively easy. The most widely used approach to generate DNA sequence data is to PCR-amplify the gene encoding RNA in the small ribosomal subunit (16S rRNA) using primers targeting generally conserved regions of the gene. The individual components of the microbiota are subsequently characterized by cloning and DNA sequencing. Analysis of large clone libraries is a bottleneck for the understanding of ecological processes in the microbiota. This chapter presents both traditional and novel bioinformatics approaches for microbiota description and analysis based on DNA sequences.

1 Introduction

Fermentation reactions are often composed of complex microbiota which are constantly changing during the fermentations (Lopes, et al. 1999). Energy sources, metabolic products, internal competition among the microorganisms, and external environments are important factors in this process. The most important, and the most difficult to understand and control, are the dynamic interactions among the microorganisms. This diversity and the dynamics pose a tremendous challenge for describing and understanding fermentation reactions.

The field of microbiota analysis has been mainly descriptive, not addressing key questions about the underlying mechanisms shaping the microbiota (Zoetendal, et al. 2004). However, the recent emergence of new molecular- and bioinformatic – tools, and the adaptation of ecological thinking from the field of macro ecology now show promise for understanding the mechanisms of interactions in the microbiota during fermentation processes. There are, however, several bottlenecks in microbiota description and understanding. The most fundamental is how to define and describe the relatedness between microorganisms. Microorganisms are small and lack sexual barriers, and most of them cannot be grown under laboratory conditions. Another important aspect is to determine differences in the microbiota

composition. In this chapter I will address the newest bioinformatics approaches to describe microbiota diversity, and how to compare differences between microbiota. Only DNA sequence-based approaches, which are among the most promising, will be covered.

2 Describing Microbiota Diversity

A microbiota is an assemblage of microorganisms with different levels of functional and evolutionary relatedness. The first step for describing the microbiota is to determine the evolutionary relations for the microorganisms present (Ludwig and Schleifer 1994). The functional, spatial and temporal relations are much more difficult to describe, and should be investigated in light of the evolutionary relations.

2.1 *Evolutionary Relatedness*

By describing the evolutionary relatedness between microorganisms, we can reflect on how the organisms have evolved. Traditionally, the description of microbial relatedness has been based on phenotypic characters such as growth requirements and morphology. The application of these approaches on microbiota samples led to low reproducibility and contradictory results. The main reasons for this are that, for most microbiota samples, we are able to grow only a minor fraction of the bacteria present, and that biochemical characters do not describe the evolutionary relatedness. It was not until the mid-1980s, with use of molecular chronometers and growth independent techniques using molecular chronometers, that we were able to start describing the real diversity of most microbiota samples (Woese 1987).

2.2 *Molecular Chronometers*

The idea of using molecular chronometers is that there is a set of genes that are present in most bacteria that are resistant to horizontal gene transfer (transfer to unrelated bacteria). The assumption is that mutations in these genes have occurred at a constant rate over time in a clockwise fashion.

The most widely used chronometer is the gene encoding the ribosomal RNA in the small subunit of ribosomes (16S rRNA). The other commonly used chronometers are gene encoding ribosomal RNA in the large subunit of ribosomes (23S rRNA), elongation factor TU and ATPase (Ludwig, et al. 1998). The difference between protein coding genes and genes encoding functional RNA is that the main selection for the protein encoding genes is on the amino acid composition; for the

functional RNAs the selection is on the nucleotide composition. The benefit of the 16S rRNA gene is that this gene is a mosaic of conserved and variable regions. Thus, the conserved regions can be used to classify distantly related bacteria, while the variable regions can be used for classification of closely related bacteria.

2.3 Alignment-based Description of Evolutionary Relatedness

The traditional way to identify the evolutionary relatedness between organisms is to first identify the homologous positions in the gene sequence through a multiple sequence alignment, and then to reconstruct the phylogeny based on the alignment. The most popular approach for making alignments is the CLUSTAL family of multiple sequence alignment algorithms. The CLUSTAL approach involves pairwise alignments, then the alignment score is used to produce a phylogenetic tree. Finally, the sequences are aligned using a dynamic alignment algorithm. CLUSTAL has been extensively optimized with respect to speed and performance (Thompson, et al. 1997). There is, however, an alternative for large datasets. This approach is based on a predefined alignment model. Such models are implemented in the major packages and databases such as ARB (Kumar, et al. 2005) and the Ribosomal Database Project II (RDPII) (Cole, et al. 2005) intended for analysis of large 16S rRNA gene datasets. The drawback is, of course, for bacteria that are distantly related to the already characterized bacteria. These cannot be aligned using a predefined model.

After the alignment is performed, the evolutionary relations between the organisms are determined by phylogenetic reconstruction. The reconstruction is based on the assumed homologous gene positions, representing the phylogenetic relations as a bifurcating evolutionary tree. There are three general approaches to generate the topology of alignment-based phylogenetic trees (Sidow and Bowman 1991). The least accurate, but by far the least computer-demanding, are the distance methods. Here, pair-wise distances are constructed for each pair of sequences in the data set; subsequently the tree that offers the best explanation for the distances is found. The most widely used approach for tree construction is the neighbor joining method. The basic concept of neighbor joining is that one starts with a star like unresolved topology. Then the nodes are identified, and the tree built. Another popular approach is the parsimony method. In the parsimony method the characters are used to identify the tree topology that explains the data with as few events as possible. The assumption is that the simplest explanation is the correct one. The final group of methods is the maximum likelihood methods. Here, a maximum likelihood estimator is used to estimate the tree topology, given an evolutionary model. This is the most accurate method, but also the slowest, enabling the analysis of relatively small sets of data.

A severe limitation with all alignment-based phylogenetic reconstructions is that they assume that the underlying alignment is correct. There are, however, no statistical tests that confirm whether this is true or not. In the phylogenetic tree construction,

on the other hand, there are methods to evaluate if the tree is correct or not. Here, there are two widely used approaches. One is the bootstrap approach, an analysis to make new datasets by randomly choosing columns from an alignment (Felsenstein 1985). The frequency of a given branch is provided as the bootstrap support. More recently, conditional Bayesian statistics have been used to directly determine the probability of the branches in phylogenetic trees (Huelsenbeck, et al. 2001). Since the use of Bayesian statistics enables a direct estimate of branch topologies, this approach is the most accurate one. The problem, however, is that the algorithms are computer-demanding. Both developments in computer speed and new algorithms now enable the calculation of the probabilities using Bayesian statistics for phylogenetic reconstruction.

An example of the alignment-based approach is shown for all 650 *Lactobacilli* sequences present in the RDPII database January 2007 (Fig. 10.1). The example

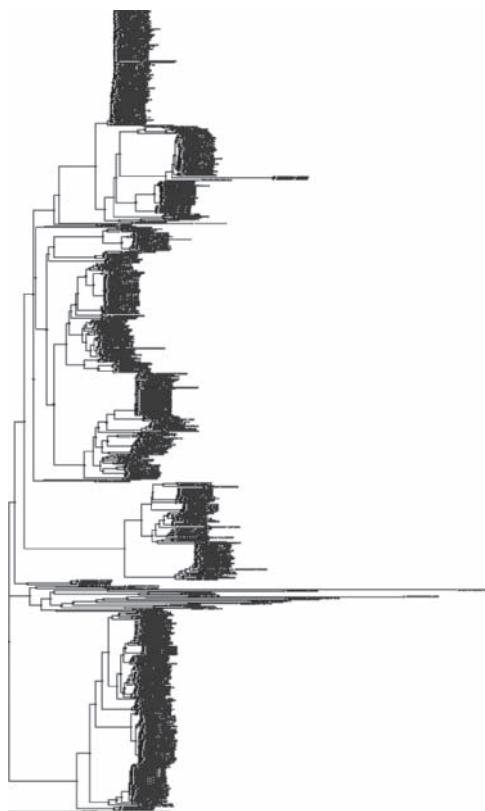


Fig. 10.1 Alignment based phylogeny for *Lactobacillus*. The tree is based on an alignment of 650 nearly full length 16S rRNA gene sequences from the RDPII database. The alignment and tree constructions were done using the alignment algorithm provided in the CLC Combined Workbench II (www.CLCbio.com)

shown here took three hours to align, while the phylogenetic reconstruction took three minutes. This approach, although providing a very detailed map of the phylogenetic relations, is limited by the fact that it is not possible to easily get an overall impression of the diversity in the dataset. As we shall see later, to use phylogenetic trees for microbiota analysis we have to make further assumptions.

2.4 Alignment Independent Description of Evolutionary Relatedness

Currently, there is an explosion in the number of DNA sequences available in public databases. There are now nearly 500,000 sequences available for the gene encoding 16S rRNA alone!. Obviously, traditional alignment and phylogenetic tree-based approaches are not suitable to analyze such large sets of data. As described above, there are two bottlenecks in doing traditional phylogenetic analysis. The first is the alignment, and the second is the tree construction. These bottlenecks must be overcome in order to analyze large datasets.

The dependence between position information and alignment is that if the position information is wrong – the alignment is out of phase – then the phylogenetic information will also be wrong. To avoid alignments one needs to untangle the positional information from the phylogenetic information. This is commonly done by searching for signature sequences. A signature sequence is a sequence with common evolutionary origin (independent of position) in different bacteria. The signature sequence approach can either be based on predefined models, or based on the dataset that is actually analyzed. A limitation with the predefined models is that if the bacteria that are analyzed are not among the bacteria used for the classification, then the model will not give a correct classification. The RDP II database uses predefined models.

An alternative alignment independent approach not requiring extensive computations is simply to transform the DNA sequences into frequencies of multimers by moving a window of a given size along the sequence (a schematic outline is shown in Fig. 10.2) (Rudi, et al. 2006). The process involves moving a window of a given size (e.g. five nucleotides) along the DNA sequence in single nucleotide steps. For each position the corresponding pentamer in a table of all possible 1024 (4^5) pentamers is added one. What is obtained by this process is a large table of multimer frequencies. In this way, the position information is untangled from the phylogenetic information. This is because it is unlikely that the same multimers in different bacteria do not have the same evolutionary origin. The multimer tables are large and contain a lot of redundant information – several multimers tell the same story. The redundant information is subsequently compressed by principal component analysis (PCA). PCA is a statistical method to identify combinations of variables (multimers) that tell the same story. An example of this method's application is given as an analysis of the same dataset as presented for the alignment dependent approach (Fig. 10.3). The multimer transformation took 1.5 minutes in this analysis,

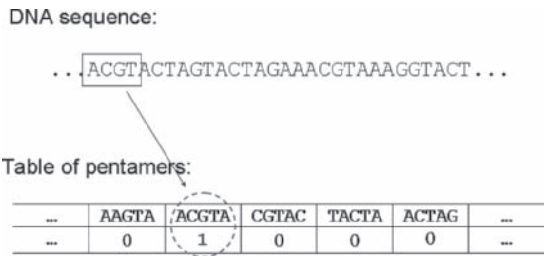


Fig. 10.2 Principle of the multimer transformation. An example of the multimer transformation using a window size of n=5. The window is slid along the DNA sequence and for each position the corresponding pentamer is increased by one. The process is repeated until all the sequences in the dataset have been transformed

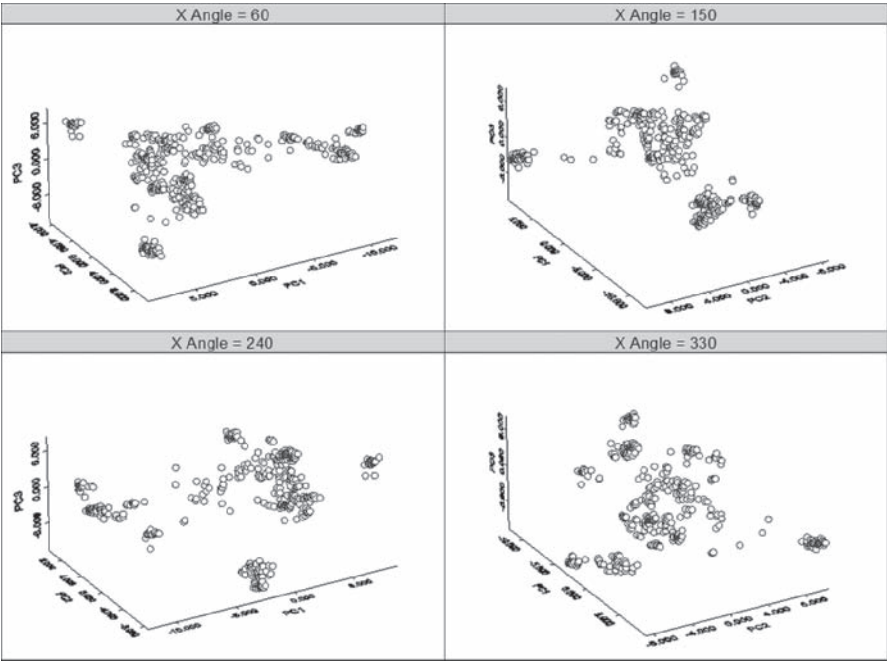


Fig. 10.3 Alignment independent phylogeny for *Lactobacillus*. The same dataset as presented in Fig. 10.1 was transformed into pentamer frequencies, and subsequently compressed by PCA using the Phylomode software. The PCA data are visualized in 3D by S+ (www.insightful.com)

while the principal component analysis took one minute. This is a considerable time saving, compared to the three hours used for the multiple sequence alignment. Using coordinates is also a more easily interpreted way to present large sets of data, compared to phylogenetic trees. It is also easily adaptable to comparisons of microbiota samples, as shown below.

3 Comparing the Microbiota Composition

Basically, there are four general dimensions in the description of the microbiota composition: spatial, temporal, evolutionary and metabolic. To address mechanisms underlying these dimensions, one needs to have tools for microbial community comparisons. The common ways to generate data from microbiota samples are outlined in Fig. 10.4. All approaches are based on PCR amplification of generally conserved genes such as the gene encoding 16S rRNA. Alternative techniques to DNA sequence-based determinations are use for DNA probes to search for specific bacteria, or profiling techniques to determine the overall composition of the microbiota. The DNA sequencing techniques are generally based on cloning of the PCR products, and sequencing of the individual components. This is the most widely used approach to determine the composition of the microbiota.

Currently, there are two common ways for DNA sequence-based microbiota comparisons. The first is based on segmenting the data into predefined categories or operational taxonomic units (OUTs) (Curtis, et al. 2002). The OUTs can be based on predefined evolutionary models such as in the RDPII database, or the dataset in itself can be used for OUT definition. Using OUTs, however, requires a segmentation of the data, and it is very difficult (if not impossible) to determine the boundaries of the OUTs. An example of the OUT approach's limits with the is given by Rudi, et al. (2007). This dataset describes the effect of mode of delivery on the infant gut microflora. The use of RDPII Library Compare did not detect the major structure in the data because the two most important bacteria were classified as the same OUT, even though they responded differently.

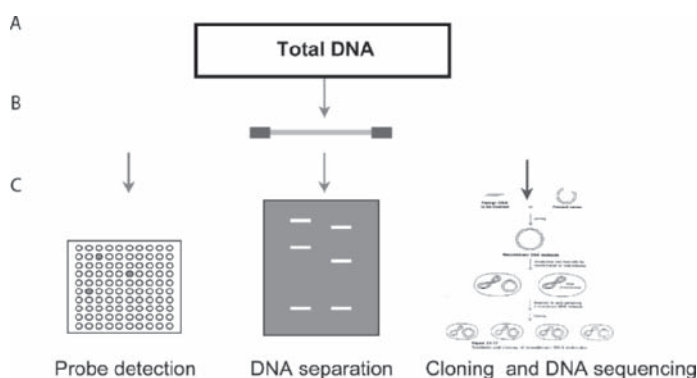


Fig. 10.4 General outline for microbiota analysis. (A) The first step in microbiota analysis is to obtain total DNA. (B) Then the DNA is PCR-amplified with PCR primers targeted to generally conserved regions in the 16S rRNA gene. (C) The bacteria present in the sample can either be detected by DNA probes, some kind of DNA separation techniques such as denaturing gradient gel electrophoresis (DGGE), or tRFLP. The most widely used approach, however, is cloning and DNA sequencing. Here the individual bacteria in microbiota are sequenced. This is the approach addressed in the current chapter

Table 10.1 Properties of Microbial Community Comparison Tools

Tool	Require alignments	Predefined groups	Phylogenetic description	Distance measure	Reference
RDPII library compare	no	yes	no	no	(Cole, et al. 2005)
∫ LIBSHUFF	yes	no	yes	relative	(Schloss, et al. 2004)
UniFrac	yes	no	yes	relative	(Lozupone and Knight 2005)
TreeClimber	yes	no	yes	relative	(Schloss and Handelsman 2006)
fLAND	no	no	yes	absolute	(Rudi, et al. 2007)

More recently, approaches for direct comparisons of phylogenetic trees have been developed (Schloss, et al. 2004). These methods are based on combining phylogenetic and frequency information to determine the relatedness between microbial communities. The challenge here is that it is difficult to determine the accuracy of the constructed trees. This will of, course, be represented in the community comparisons in untangling differences due to phylogenetic differences, and differences due to inaccuracies in the tree construction.

Recently an approach for comparing microbiota samples that is based on phylogenetic information, but avoids tree construction, has been developed (Rudi, et al. 2007). This approach is based the same multimer transformation described above. In the microbial community comparisons, the densities for the different microbiota samples are compared. This approach is efficient with respect to Central Processing Unit (CPU) usage, as demonstrated in an analysis of the distribution of human gut bacteria, where we analyzed 11,831 bacteria from three individuals from a human gut library.

A summary of commonly used approaches for microbial community comparisons are presented in Table 10.1.

4 Future Developments

Data generation remains a challenge with future microbiota analysis . One needs large datasets with accurate information about the microbiota composition. This is currently done by cloning and DNA sequencing (see Fig. 10.4). Traditional cloning and DNA sequencing, however, is too labor intensive to generate sets of data that are suitable for large-scale microbiota analysis. The emergence of novel and high-throughput DNA sequencing techniques, however, shows great promise with respect to high-throughput generation of data (Metzker 2005).

With good descriptive data of the microbiota in hand, these can be used in ecological modeling to determine the underlying ecological mechanisms. As described in the introduction, adapting ecological tools from traditional ecology would greatly benefit the field of microbial ecology. With the understanding of spatial and dynamic interactions the use of fermentation in food production would benefit, enabling more rapid food product developments, better quality control and safer products. The downside, however, is that these developments are just in its infancy, and both technical and analytical challenges need to be overcome before advanced microbiota analysis can be used in practice.

References

- Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., Garrity, G.M., Tiedje, J.M., 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* 33, 294–296.
- Curtis, T.P., Sloan, W.T., Scannell, J.W., 2002. Estimating prokaryotic diversity and its limits. *Proc. Natl. Acad. Sci. USA* 99, 10494–10499.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791.
- Huelsenbeck, J.P., Ronquist, F., Nielsen, R., Bollback, J.P., 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294, 2310–2314.
- Kumar, Y., Westram, R., Behrens, S., Fuchs, B., Glockner, F.O., Amann, R., Meier, H., Ludwig, W., 2005. Graphical representation of ribosomal RNA probe accessibility data using ARB software package. *BMC Bioinformatics* 6, 61.
- Lopes, M.F., Pereira, C.I., Rodrigues, F.M., Martins, M.P., Mimoso, M.C., Barros, T.C., Figueiredo Marques, J.J., Tenreiro, R.P., Almeida, J.S., Barreto Crespo, M.T., 1999. Registered designation of origin areas of fermented food products defined by microbial phenotypes and artificial neural networks. *Appl. Environ. Microbiol.* 65, 4484–4489.
- Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228–8235.
- Ludwig, W., Schleifer, K.H., 1994. Bacterial phylogeny based on 16S and 23S rRNA sequence analysis. *FEMS Microbiol. Rev.* 15, 155–173.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M., Schleifer, K.H., 1998. Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* 19, 554–568.
- Metzker, M.L., 2005. Emerging technologies in DNA sequencing. *Genome Res.* 15, 1767–1776.
- Rudi, K., Zimonja, M., Kvenshagen, B., Rugtveit, J., Midtvedt, T., Eggesbo, M., 2007. Alignment-independent comparisons of human gastrointestinal tract microbial communities in a multidimensional 16S rRNA gene evolutionary space. *Appl. Environ. Microbiol.* 73, 2727–2734.
- Rudi, K., Zimonja, M., Naes, T., 2006. Alignment-independent bilinear multivariate modeling (AIBIMM) for global analyses of 16S rRNA gene phylogeny. *Int. J. Syst. Evol. Microbiol.* 56, 1565–1575.
- Schloss, P.D., Handelsman, J., 2006. Introducing TreeClimber, a test to compare microbial community structures. *Appl. Environ. Microbiol.* 72, 2379–2384.
- Schloss, P.D., Larget, B.R., Handelsman, J., 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. *Appl. Environ. Microbiol.* 70, 5485–5492.
- Sidow, A., Bowman, B.H., 1991. Molecular phylogeny. *Curr. Opin. Genet. Dev.* 1, 451–456.

- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882.
- Woese, C.R., 1987. Bacterial evolution. *Microbiol. Rev.* 51, 221–271.
- Zoetendal, E.G., Collier, C.T., Koike, S., Mackie, R.I., Gaskins, H.R., 2004. Molecular Ecological Analysis of the Gastrointestinal Microbiota: A Review. *J. Nutr.* 134, 465–472.

Chapter 11

Role of Bacterial ‘Omics’ in Food Fermentation

Monique Zagorec, Stéphanie Chaillou, Marie Christine Champomier-Vergès, and Anne-Marie Crutz – Le Coq

Abstract For most lactic acid bacteria (LAB) commonly used in the manufacturing of fermented foods of plant or animal origin, at least one whole genome sequence is now publicly available. This huge amount of new information greatly helps in understanding the complex mechanisms that were used empirically by humans to produce and preserve a large part of their food for millenaries, and that started to be scientifically investigated only a few decades ago. Genomics provides new tools to monitor, control, modify or improve such products. The post-genomic era allows, for the first time, a molecular dissection of the fermentation process in its entirety. Together, genomics and post-genomics approaches considerably accelerate time scale by bringing a deluge of data and representing a new challenge for food microbiologists, raising the possibility of having valuable information that can be in the food application.

1 Introduction

The first complete sequence of a bacterial genome, *Haemophilus influenzae* was published in 1995 (Fleischman, et al. 1995), thus delivering for the first time, all the genetic information encoded by the chromosome of a known bacterium. Only a decade later, more than 400 complete sequences of bacterial genomes are available (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1>) and even more are in progress to be completed (<http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi>). What can we expect from this genomic era rising? Ten years after sequencing of the *H. influenzae* chromosome, a commensal resident of humans responsible for respiratory tract infections, have we progressed to the point of curing or avoiding the infections it causes? We do not. However, the genomic and post-genomic approaches promise important changes that will contribute to a better knowledge and control of many complex processes. The two major consequences of this wealth of data is, first, the opportunity to better understand and predict the role of bacteria and, second, the development of many new technologies that enable us to experimentally verify hypotheses made from genome data mining, and to monitor

complex systems. In the field of the agro industry, such as the fermentation of food products, one can expect that the genomic and post-genomic era will have a deep impact.

Genomic analyses (e.g., analysis of all the gene sequences that an organism possesses, also called “*in silico*” analysis, and genome data mining) lead to predict many of the potentialities of a bacterial species. By genome comparison of bacteria from the same phylogenetic group or occupying the same environment, it becomes possible to predict what occurs in a particular environment or how bacterial genomes, within a phylogenetic group, are evolving. At the same time, several “post-genomic” approaches are being developed, reflecting the huge progress made in the techniques of several methods of analysis (as examples: improvement of two-dimensional electrophoresis, HPLC, and of mass spectrometry performance, design of DNA-chips) and whose efficiency to produce biologically meaningful information was alimented by the availability of the genome data. For instance, proteomic analyses (analysis of all proteins produced by an organism at a certain time or in a certain condition) become much more informative once the genome sequence of an organism is known, because identification of the proteins of interest is made easier and because genome data help to predict their function. Transcriptomic analyses (analysis of all the transcripts synthesized by an organism) of a bacterium, through the use of DNA-chips or DNA-arrays are also rendered possible after completion of the genome sequencing of this bacterium. Metabolomics (study of all the metabolites produced by an organism) is still in development and not yet fully exploited due to the lack of accurate miniaturized methods and relevant high throughput technology, and because of the difficulty in making direct links between the genome data and the metabolome data. Many new terms were introduced due to these recent developments, such as secretome, fluxome and physiome, and some are already obsolete while many more are still to be invented. Thus, global terms of “omes” and ‘omics’ reflect a new way of studying biological data through a combination of several global approaches. These global approaches are now applicable in fields that concern the production of fermented food products.

The genomes of bacteria used as models by scientists, like *Escherichia coli* and *Bacillus subtilis*, as well as those of many bacterial species that are pathogenic for humans, animals, or plants, have been sequenced several years ago. For some of those, the genome of several strains belonging to the same species have been sequenced, revealing a high genomic diversity even in well defined species. More recently, the genomes of bacteria important for food fermentation were also sequenced. Indeed, almost all the genomes of LAB involved in the fermentation of many products derived from milk, meat and several vegetable products, and those of several probiotic LAB are now available. This enables scientists to predict the potentialities of the various species involved in food processing. In addition, ‘omics’ have also been initiated for these bacteria and should offer better knowledge and control of the fermentation processes involving LAB.

The major impacts of these recent developments are exposed below. First, the genome data mining will bring a new vision of LAB, their potential uses and their evolution. Those bacteria, that are certainly old companions of humans, have been

used by us since millenaries and we will use them even more efficiently. Second, the post-genomic approaches that are now arising will help to better monitor and control the processes. Third, we can expect new tools to be developed for a better evaluation of the quality and evolution of fermentation productions and an easier traceability of the products.

2 Genomics of LAB: Toward an Improvement and a Refreshment of Our View on a Versatile Group of Food Bacteria

Scientists need to practice a precise, concise and common language. Taxonomy procures this as it defines and classifies the organisms we are living with into groups. The LAB group is an interesting example in that sense, as its definition was initially based on phenotypic traits: LAB are producing lactic acid from sugar fermentation. Recently, the criteria that define a bacterial species have been discussed as a consequence of the new features that emerged from genome sequences and genome comparisons (Lan and Reeves 2000; Coenye, et al. 2005; Doolittle and Papke 2006). In this context, the LAB group must be reconsidered. A picture of uniformity has often been given to the different species composing this group. Undoubtedly, they share many metabolic traits, especially those related to the degradation of sugars to lactic acid, a process involved in the biopreservation of many food products. Perhaps the similarity between LAB species stops here. There is, indeed, a much broader scale of physiological variations and numerous species are associated with a variety of unrelated ecological niches. In the last few years, it has become particularly obvious that these variations are reflecting a natural genetic variation among LAB species. We are just beginning to collect this knowledge from the deluge of data engulfing us from genome sequencing projects.

To date, the genome sequence of 16 LAB species is publicly available (Table 11.1), and for some species the genome sequence of several strains has been determined. Most of the sequenced species belong to the phylogenetic group of lactobacilli (10 species). This 'special fondness for lactobacilli' as expressed once by Tannock (Tannock 2004), is not only an indirect consequence of their economic importance in food fermentation, but also the result of the natural diversity of this genus. Indeed, with more than 80 different recognized species, lactobacilli undoubtedly account for the largest genetic diversity among LAB in comparison to lactococci (5 species), leuconostocs (11 species) and pediococci (6 species), the latter being closely related to lactobacilli (Hammes and Hertel 2004). Furthermore, although many sequencing projects were launched independently from several laboratories worldwide, it is a fortunate coincidence that a species of almost every major phylogenetic sub-group, representing various environmental niches and fermented food products, has been analyzed. This ample coverage of the lactic acid microbial world and the wealth of information it is providing offers current food microbiologists an unprecedented opportunity to improve their view on these bacteria, and their role in food fermentations.

Table 11.1 Publicly Available Complete Genome Sequences from Lactic Acid Bacteria

Species	Strain	Size (Mb)	Ecological specificity or food product specificity	Reference
Low G+C percent				
<i>Lactobacillus acidophilus</i>	ATCC700396	2.0	Gastrointestinal tract of animals, vagina	Altermann, et al. 2004
<i>Lactobacillus brevis</i>	ATCC367	2.3	Ubiquitous in plant-derived products (silage, beer, wine, coffee)	Makavora, et al. 2006
<i>Lactobacillus casei</i>	ATCC334	2.9	Ubiquitous in milk- and plant-derived products, gastrointestinal tract of animals	Makavora, et al. 2006
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	ATCC11842	1.9	Yogurt and other related milk-fermented products	van de Guchte, et al. 2006
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	ATCCBAA365	1.9	Yogurt and other related milk-fermented products	Makavora, et al. 2006
<i>Lactobacillus gasseri</i>	ATCC33323	1.9	Gastrointestinal tract of animals, vagina	Makavora, et al. 2006
<i>Lactobacillus johnsonii</i>	NCC533	2.0	Gastrointestinal tract of animals, vagina	Pridmore, et al. 2003
<i>Lactobacillus plantarum</i>	WCFS1	3.3	Ubiquitous in plant-derived products (silage, wine, cocoa, sauerkraut, olives)	Kleerebezem, et al. 2003
<i>Lactobacillus sakei</i>	23K	1.9	Meat- and fish-derived products, starter for Sausages in Europe	Chaillou, et al. 2005
<i>Lactobacillus salivarius</i>	UCC118	2.1	Saliva, gastrointestinal tract of animals	Claesson, et al. 2006
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	IL1403	2.3	Ubiquitous in milk- (especially cheese) and plant-derived products	Bolotin, et al. 2001
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	SK11	2.6	Ubiquitous in milk- (especially cheese) and plant-derived products	Makavora, et al. 2006

(continued)

Table 11.1 Publicly Available Complete Genome Sequences from Lactic Acid Bacteria (continued)

Species	Strain	Size (Mb)	Ecological specificity or food product specificity	Reference
<i>Lactococcus lactis</i> subsp. <i>cremoris</i>	MG1363	2.5	Ubiquitous in milk- (especially cheese) and plant-derived products	Wegmann, et al. 2007
<i>Leuconostoc mesenteroides</i>	ATCC8293	2.0	Fruit- and vegetable-derived products	Makavora, et al. 2006
<i>Oenococcus oeni</i>	ATCCBAA331	1.8	Wine	Makavora, et al. 2006
<i>Pediococcus pentosaceus</i>	ATCC25745	1.8	Ubiquitous in milk- and plant-derived products, starter for Sausages in US	Makavora, et al. 2006
<i>Streptococcus thermophilus</i>	LMG18311	1.8	Yogurt and other related milk products	Bolotin, et al. 2004
<i>Streptococcus thermophilus</i>	CNRZ1066	1.8	Yogurt and other related milk products	Bolotin, et al. 2004
<i>Streptococcus thermophilus</i>	LMD-9	1.8	Yogurt and other related milk products	Makavora, et al. 2006
High G+C%				
<i>Bifidobacterium longum</i>	NCC2705	2.3	Gastrointestinal tract of animals	Schell, et al. 2002

2.1 Genomic Diversity and the Evolution of LAB

Our efficient use of the genomic information is, of course, highly dependent on the way one will decide to integrate and analyze the massive flow of 'omics' data being generated. For instance, the goals of the genome projects, as well as the type of approach subsequently used for the analysis, were often different. It is certainly rational to say that scientists investigated genomes of LAB with a particular view to their commercial exploitation in mind. This was ordinarily associated with several objectives such as food improvement and functionality, understanding bacterial adaptation to specific food products, resistance and survival of starter strains to food processes, roles of antimicrobials and food safety, host-microbe interactions for the development of probiotics or functional food. As a logical result of these differences, both the annotation process of genomic data and the extraction of comprehensive scientific information from these data have been highly dependent on the choice of annotation tools, and on the level at which the curators were taking the information into account. Whatever the level of success encountered in this task, it appeared that these *in silico* genomic studies were a 'horn of plenty' for studying genomic evolution of LAB. By offering a closer look at the main scientific messages often emphasized in the recent LAB genome's publications, it is clear that studies on the evolutionary relationship of the different LAB species have been one important output of the sequencing projects, although this was unplanned.

Comparative analysis of LAB genomes has provided interesting insight into their important genetic diversity and into the high divergence in their genome content. For instance, whereas LAB genomes harbor between ~1,700 to ~3,200 protein-encoding genes covering altogether up to 3,200 COGs (Clusters of Orthologous Genes) for the 15 low-GC percent species, only 20 percent of these COGs seem to represent the conserved core of genes (Makarova, et al. 2006). When lactobacilli alone are considered, the percentage of the conserved core of genes may rise but only to 40 percent (Boekhorst, et al. 2004; Chaillou, et al. 2005). The remaining non-conserved genes are, thus, reflecting the wide genetic arsenal in relation to the variety of phenotypic, physiological and ecological properties harbored by LAB. Species from the same phylogenetic subgroup such as *Lactobacillus johnsonii*, *Lactobacillus acidophilus*, *Lactobacillus gasseri* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, display rather conserved genome organization and gene synteny (Klaenhammer, et al. 2005; van de Guchte, et al. 2006), but this conservation is rapidly lost when the comparison is carried out between *Lactobacillus* species from different phylogenetic subgroups (Boekhorst, et al. 2004). In general, the co-linearity is limited to small gene clusters and the chromosome of LAB is usually showing considerable rearrangements. From these analyses it became evident that pediococci definitely belong to the lactobacilli phylogenetic group, albeit they have a misleading phenotypic cocci-like shape (Makarova, et al. 2006). Similarly, enterococci may be more related to lactobacilli than previously suspected and lactobacilli are much more different from lactococci in their genome organization than previously estimated. It is now obvious that the availability of complete LAB genomes

and the use, in the future, of phylogenomic analysis will enable a better understanding of the processes which may be involved in shaping the chromosome of individual LAB species.

The scientific community of microbiologists has now acknowledged the concept that the evolution of bacterial chromosomes is mostly the result of two conflicting mechanisms: genetic novelty and innovation via horizontal gene transfer, which often allows bacteria to acquire new ecological fitness, but leads to more chromosomal instability; and so-called 'reductive evolution' via gene decay and gene loss of no longer necessary functions, thereby restoring a more efficient chromosomal organization. Some clues have already emerged from few comparative genomic approaches between LAB genomes, and it is interesting to note the high heterogeneity of evolution rates within them (Boekhorst, et al. 2004; Chaillou, et al. 2005; Hols, et al. 2005; Klaenhammer, et al. 2005; Kok, et al. 2005; Makarova, et al. 2006). Some species have been prone to considerable gene loss from a more ubiquitous ancestor, demonstrating that many lactobacilli have evolved toward a specialization of nutrient-rich environments such as food products. This reductive evolution is noticeable in *Leuconostoc*, *Oenococcus*, *Pediococcus* and in the yogurt starters species *Streptococcus thermophilus* and the *Lb. delbrueckii* subsp. *bulgaricus* group. In particular, *St. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* exhibit a high percentage of pseudogenes (10 percent for each in comparison to 1 percent to 2 percent in most bacterial genomes) suggesting an active ongoing process of gene decay (Bolotin, et al. 2004; van de Guchte, et al. 2006). This substantial gene inactivation, that is not yet followed by gene loss, is presumably indicative of a recent ecological evolution, which most likely started with the rise of mammals 60 millions years ago (Bolotin, et al. 2004). In some other species there is a clear emergence of many new genes via gene duplication and horizontal gene transfer. This observation is clear for species with larger genomes and with more versatile ecological preferences such as *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus brevis* and *Lactococcus lactis* (Makarova, et al. 2006), but also for more ecologically specialized organisms such as *Lactobacillus sakei* (Chaillou, et al. 2005) and *Lactobacillus salivarius* (Claesson, et al. 2006). However, we must not forget that only a few strains were sequenced, and the choice made for the sequencing projects might have introduced a bias. Indeed, some strains selected as starters or cultivated for a long time may have evolved even more recently than what we suspect, and the few sequenced samples may not represent their species.

A new and interesting observation was made for *Lb. salivarius* genetic diversity. In this species the 240 kb megaplasmid carried, shows significant strain-dependent variations, constituting an important flexible genetic complement to the chromosome. Important intraspecies variations were also observed in *Lb. plantarum* (Molenaar, et al. 2005) and *Lb. sakei* (our unpublished data), and are clearly indicating that we are just beginning to learn about the variable genetic make-up of LAB species and the evolutionary relationship that exists between species and strains. Can we conclude that our current classification of LAB species is not satisfactory? This classification may not be so useful after all, since it ignores most of the genetic

diversity between strains of the same species and the genetic diversity between already recognized species.

The case of the 'species and strain definition and concept' is not a brand new problem. As summarized recently by Doolittle and Papke – "Whether or not bacteria have species is a perennially vexatious question" (Doolittle and Papke 2006). But recent genomic studies on a wide range of bacteria tell us that the within-species variability can be enormous and it is even speculated that the core genome (core set of genes conserved in most strains of the same species) can be, in some cases, narrower than the pan genome (auxiliary set of genes variable between strains of the same species). This is, of course, a huge modification of our view of the bacterial species concept because it dramatically drops the sharpness of species boundaries. It offers, on the other hand, a more coherent genetic and/or ecological model for bacterial diversification and adaptation. The idea of studying the intraspecies biodiversity of LAB is barely awakening. While DNA chips are becoming available for some species and techniques, such as subtractive hybridization for fishing out strain-specific genes, can be easily applied, DNA microarray-based genotyping might radically change our view on the microbial world of LAB.

2.2 Genomic Diversity and Microbial Ecology of Food Products

What sort of changes could our knowledge on the genomic biodiversity of LAB provide to the field of food fermentation or use of probiotics? First, it is important to remember that LAB usually live and develop within complex microbial communities such as the digestive tract of animals or traditionally fermented food products. The intrinsic genomic diversity of LAB most likely reflects the need for a species to adapt and survive to such harsh and highly competitive environments and, eventually, to respond to the many fluctuations or variations that might be encountered (modification of the animal diet, changes in the gut microbiota or succession of various food or feed technological processes). Therefore, studies on genomic diversity are, simultaneously, a need and a tool. It is needed to acquire an accurate and better understanding of the community/population dynamics of LAB in these complex environments and of the role of microbial ecology in food fermentations. Without this knowledge, both the development of probiotics (behavior and efficacy of allochthonous probiotic strains versus autochthonous strains) and our chance to better manipulate or master traditional fermentation processes will be hindered. A comprehensive analysis of the genomic diversity may also serve as an efficient tool to explore the microbial ecology of food products. One pioneering work has already been published on the use of microarrays for monitoring the diversity of LAB on traditional Korean fermented food products (Bae, et al. 2005). In this trial, the biodiversity was quantified, at the species level, by using genomic DNA hybridization. However, one may envision the use of gene-based DNA microarrays to fingerprint the ecological diversity at the strain or genotype level. Such possibility would pave the way to tremendous applications in the field of food

fermentation, such as the opportunity to better characterize worldwide culture collections of LAB, or to correlate food product typicity to specific microbial communities (monitoring and traceability).

2.3 Genome Diversity and Adhesion Properties of LAB: Comparison of Food and Intestinal Species

Emphasis has been given to the characterization of cell surface proteins from lactobacilli genomes. This particular attention was motivated because of the possible role of cell surface proteins in host-microbe interactions, therefore, in the probiotic potential of these organisms. From these studies it has become evident that lactobacilli species show a rather unique repertoire of these cell surface proteins, indicating a functional category with very weak overall conservation. Three important families of cell surface proteins were detected in lactobacilli and their specific distribution in the different species revealed interesting ecological features. An important family includes proteins with a mosaic structure of Mucin Binding (MUB) domains which mediate adhesion to mannose moieties of mucin (Boekhorst, et al. 2006). Since the MUB proteins most likely constitute a first and major contact point between the surface of lactobacilli cells and mucins (glycosylated proteins) which are covering the epithelial cells of the intestine, it is not surprising that these proteins are highly abundant in intestinal lactobacilli, but rare or absent in food-related lactobacilli. The second family includes proteins encoded by a novel type of gene clusters called CSC (Cell surface Complexes) (Chaillou, et al. 2005; Siezen, et al. 2006). Contrary to MUB proteins, the CSC complexes are more abundant in food-related LAB than in intestinal lactobacilli, and are even found in food-borne pathogens such as *Listeria monocytogenes*. These proteins presumably form structural complexes on the surface of lactobacilli in which some components may be tightly bound to the cell wall, whereas others may be more loosely bound and could be released from the cell depending on the environmental conditions. The largest proteins identified in the various CSC display large variable domains, showing similarities to proteins such as lectin/concavalin, immunoglobulin-like binding domains, bacterial adhesins, hemagglutinin, invasins and fibronectin-binding domains, and are believed to adhere and degrade numerous surface polysaccharides found in either animal or plant-derived food products. They may also promote auto-aggregation or co-aggregation with other bacterial cells and, therefore, may play an important role in the interactions with other microbes (Schachtsiek, et al. 2004). Finally, the third family includes the so-called Aggregation Promoting Factors (APF), which are S-layer-like proteins. The APF are paralogous modular proteins found in various amounts and with various combinations among *Lactobacillus* species (Åvall-Jääskeläinen, et al. 2005). These proteins mainly carry out a structural role in the shape of the bacteria and act as a physical barrier to extra-cellular components. However, they are also known to mediate adhesion to many molecules such as collagen, or to be involved in cellular aggregation. Functional studies of the

S-layer-like proteins of *Lb. acidophilus*, *Lb. brevis* and of the APF proteins of *Lb. gasseri* have even shown that they can sometimes be expressed differentially during environmental changes, or sometimes expressed in combination or even as two different surface layers. So far and as described above, each LAB species possesses a unique combination of these three important surface proteins families, rendering the prediction of its adhesion properties rather difficult.

This reflects our limited understanding of the adhesion process and the behavior and interactions of LAB in their environmental niches. Of course, this point is far from trivial in the field of food fermentation. Indeed, many fermented food products are made of a complex matrix, quite often solid, and therefore considerably different from the common laboratory broth media. This implies that important mechanisms of colonization are utilized by LAB to ensure an efficient fermentation process. There is still a paucity in our knowledge of this area of the LAB physiology. Biofilm formation of LAB, for instance, has not yet been an important field of scientific investigation, although it is presumably an important parameter in some fermented products. Therefore, aside from adhesion properties, functional studies of these cell surface proteins might also help to elucidate how bacteria co-aggregate, and how they colonize solid food matrices, and thus will contribute to the food innovation cycle. Moreover, many food bacteria enter the gastrointestinal tract and can survive through it, and particularly the *Lactobacillus* genus encompasses species that are either food-borne species, commensal species of the human or animal intestinal tract, or both. Thus, a deeper analysis of the cell surface proteins, specific for each type of bacteria, should help to understand their involvement in the colonization of the various niches.

3 From Genomes to Food Fermentation: The Impact of ‘Omics’ and Modeling Strategies on the Understanding of Metabolism

Besides *in silico* genome analysis, experimentations such as proteomics have been used to explore physiology of food fermenting bacteria, especially those from the LAB group. Main emblematic bacteria of this group have been studied using this methodology, and literature provides references for bacteria originating from milk, meat, wine or vegetal fermented food products. Along with progression of whole genome sequencing projects in this group of bacteria, transcriptomics is also becoming a tool for researchers. However, so far, these studies have been mainly restricted to small scale laboratory conditions. Although they provided valuable information on the physiology of bacteria (mainly on stress response and adaptation to various environmental conditions), one can now reasonably envision further and future utilizations in new challenges emerging from food fermentation. Metabolomic studies are also being developed on LAB metabolisms involved in food processing. This latter omics approach is less developed experimentally, but rather well documented through genome *in silico* analysis and metabolic pathways modeling and reconstruction.

Such developments rely largely on data mining, which is one of the major challenges for this post genomic era. Indeed, understanding the information emerging from these new tools requires integration of data from many different sources. However, such development requires increased bioinformatic and database resources.

3.1 *In silico Analysis and Metabolic Modeling*

Genome annotation is the first step toward understanding the function of genes, and this type of analysis can be used for predicting the metabolic potentialities of LAB. In principle, such genomics-based knowledge may lead to a better prediction of fermentation behavior of strains or to the detection of target genes encoding new metabolic pathways. Then it is tempting to believe that experimental functional genomic strategies will be easily applied to functional food innovation or to the design of new probiotics.

However, knowledge acquisition out of the exploitation of the genomic data is still a challenging task, mainly because the use of genomics to understand metabolism does not solve all the problems. Ironically, it even creates new ones.

One of these problems, as mentioned earlier, is the quality of the annotation data, but also the lack of a database for specific LAB metabolic pathways from which more accurate predictions can be made. Constructing a comparative metabolic database for all LAB species would be part of the solution to this problem. Such a database has already been constructed for *Lb. plantarum* and has helped in comparing *in silico* predictions with those from growth experiments (Teusink, et al. 2005). On a larger scale, however, defining strategies for a well-curated LAB species-wide database would be essential if the aim is to be useful for a wide scientific community. The genomic-based approaches are also facing difficulties relating to the gaps often found between what is already known and what could be predicted. Whole genome annotation has also taught scientists the limitation of discovering new metabolic pathways from genes of unknown functions. Comparative genomic studies are often helpful in such a task, but usually cannot overcome the difficulty of building models. For instance, reconstruction of metabolic pathways from genome data sometimes tells a different story (or at least a more complicated story) than what had been described from biochemical or physiological experiments. Such inconsistencies reflect our difficulty to evaluate the complex relationships existing between genes, proteins and metabolic reactions.

Several examples of unprecedented metabolic reconstruction in LAB were described recently from genome data showing the impact of manual annotation. For instance, analysis of the *Lb. plantarum* genome has revealed suitable gene candidates for molecular functions corresponding to missing links in some metabolic pathways (reactions for which no coding sequences had been assigned) such as vitamin biosynthesis (Teusink, et al. 2005). This study is a good illustration of how, sometimes, automatic assignment of biological function to a gene can be misleading

and, thereby, what sort of strategies could be applied to identify analogous genes (non-homologous genes belonging to the same function) belonging to known pathways. In a different way, it is often difficult to predict functional roles to paralogous genes (group of homologous genes encoding similar molecular functions). Analysis of nucleosides scavenging pathways encoded by *Lb. sakei* genome has revealed how functional redundancy might reflect an important metabolic network, presumably required for environmental survival (Chaillou, et al. 2005). In this case enzymes that are normally assigned to nucleosides biosynthesis in LAB were also considered to play an important role in degradation and energy production. These studies are examples of how exhausting and tricky the search for missing enzymes or pathways can be. It is also interesting to note how the glycolytic pathway, the high flux route from sugar to lactic acid and the paradigm, per se, of ATP production from all LAB species, has revealed striking differences once several of the LAB genomes were analyzed. Most of these differences are present at key regulatory feed-back reactions such as the reverse conversion of fructose 1,6-biphosphate to fructose 6-phosphate by fructose 1,6-biphosphatase, or at the conversion of pyruvate to phosphoenolpyruvate (not always performed in LAB) which seems to be carried out with either pyruvate phosphate dikinase or pyruvate water dikinase. Some species have also kept (or acquired) specific shortcuts or bypasses, such as the non-phosphorylating NAD⁺-dependent glyceraldehyde 3-phosphate dehydrogenase in *St. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus*, or a possible methylglyoxal bypass in *Lb. sakei*. The genome sequences of both *Lb. plantarum* and *Lb. salivarius* have also revealed that these two organisms theoretically have a functional hexose monophosphate pathway, whereas only the Embden-Meyerhof-Parnas pathway or the xylulose 5-phosphate phosphoketolase pathway were previously reported to exist in LAB. It is speculated that these important differences are reflecting specific regulations of the glycolytic flux, possibly in relation to the ecological niche of the different species. This observation is of particular interest as we now look more closely at industrial fermentation processes.

In this context, genome-scale modeling methods are perhaps the most promising approaches to improve the industrial exploitation of LAB (Teusink and Smid 2006). These methods are a combination of mathematical modeling techniques (kinetic models and metabolic control analysis) with functional-genomics data (transcriptomics, proteomics and metabolomics). These studies are expected to give insights on the molecular basis relevant to stress-response adaptation mechanisms, nutrients auxotrophies, and the kinetic characteristics of biotechnological-important metabolic pathways relating to e.g., lactic acid, flavor or exopolysaccharides productions. Sensitivity of these methods is a major issue and the validity and accuracy of these strategies would, of course, require some important set-up: (i) a prior analysis of genome data to carefully fill the gaps (missing links) of metabolic pathways as described above; (ii) ensuring that metabolism is stopped as quickly as possible before any experimental measurement of either mRNAs, proteins or metabolites concentrations is carried out (Hollywood, et al. 2006). Unfortunately, quantitative measurement in a reproducible and robust way is not yet available for all metabolites and all types of proteins. Functional 'omics' have raised our experimental need to a higher standard level, and it must be said, to a higher average cost.

3.2 *Metabolic Engineering and in vivo Metabolism*

Metabolic engineering of bacteria consists of modifying their metabolism to enhance their ability to produce target molecules, or to diminish the level of unwanted ones. The objective may be the improvement of starter strains for either optimizing a process or rendering the product different (e.g., new texture or taste) safer or more valuable. To date, many examples of metabolic engineering of LAB, carried out by classical techniques, have been published. However, as we await the repercussions of modeling strategies, global technologies should play a major role in achieving rational metabolic engineering since this does not often rely on modification of a single gene, but on complex bacterial metabolic networks which tend to resist perturbation (Park, et al. 2005). A few examples, cited below, may help the reader to understand in which directions the future 'omics' studies might improve food fermentation processes.

Recently, the improvement of knowledge about glucose metabolism of *L. lactis* led to the construction of a strain excreting glucose while fermenting lactose, the main sugar present in milk, with two possible applications: the manufacture of products with lower levels of lactose, for lactose intolerant individuals and sweetening of dairy products by natural fermentation through *in situ* production of glucose (Pool, et al. 2006). In this case transcriptomics was used to discover a previously unknown additional transporter for glucose, which could then be inactivated for complete blocking of glucose metabolism. Techniques of fluxomics (in vivo NMR) demonstrated the expelling of the glucose moiety of lactose into the medium (Pool, et al. 2006).

In the field of unwanted biological products in food, one can think of biogenic amines produced by bacteria. As mentioned by Pessione, et al. (2005) "all fermented foods are subject to the risk of biogenic amine contamination." Several enzymes catalyzing the decarboxylation of certain amino acids leading to the production of toxic amines are known. Among all amines tyramine and histamine, derived from tyrosine and histidine respectively, are the most toxic for the consumer. These authors used proteomics with two *Lactobacillus* strains to evaluate, in a global approach, which proteins showed a biosynthesis modulation during the production of biogenic amines. This example offers a broader understanding of the functions involved in such a production, in addition to the previously described decarboxylases. This might be an important issue to consider in the selection of starter strains. Also, it showed the physiological importance of biogenic amine production, which can occur by several pathways, and can serve energetic purposes for the cell.

3.3 *Applying 'Omics' in situ: Benefits and Difficulties to Understand Bacteria During Food Processing*

Most of the published studies aimed at understanding the physiology of microorganisms used as cell factories for the production of molecules or in different types

of fermentation processes. A major challenge for the 'omics' is their use to directly investigate processes ongoing in food samples. So far, the most representative 'omics' studies performed directly in food matrices have mainly used proteomics and, to a lesser extent, transcriptomics. The major reason for this is the very recent emergence of the application of those techniques to complex systems such as food. Some examples of using experimental approaches performed in food matrices or in media with composition comparable to food matrices are given below. Most of the literature in this field refers to milk products. This is likely due to the history of experience and knowledge about these products, but also because of the possibility to generate model matrices, either sterile or contaminated at very low level, allowing to investigate the role of inoculated strains on those models. For instance, identification of proteins present during cheese ripening and resolution of their origin and function could be achieved by a proteomic approach. This study led to the conclusion that casein degradation during ripening of Emmental was due to the fact that peptidases were produced by *St. thermophilus* and *Lactobacillus helveticus*. This was made possible by the use of milk, which was partially sterilized, and contained a very low level of endogenous flora (10^2 per g, representing 10^{-7} that of the natural flora) and did not interfere with the starter cultures used in the study (Gagnaire, et al. 2004). In another example, Larsen, et al. (2006) investigated, by a proteomic and transcriptomic approach, which genes and proteins of *L. lactis* were specifically regulated by the growth phases. This was monitored in a chemically defined medium and also in reconstituted skim milk. A good correlation was observed at the protein and transcript level. Similarly, Derzelle et al. (2005) determined the proteins produced by *St. thermophilus* during its growth in skim milk, by comparison to cultivation in a laboratory medium. This revealed that growth in milk resulted in an unexpected, important induction of the pyruvate formate lyase that converts pyruvate into formate and acetyl-coA, and the authors suggest that the formate then produced would be metabolized by the cells rather than excreted. Interestingly, a similar approach performed with another dairy lactic acid bacterium, *L. lactis*, pointed out another behavior (Gitton, et al. 2005), showing that the results found with one lactic acid bacterium may be different in other species. A few other examples result from transcriptomic studies. For instance, it was shown that *L. lactis* adaptation to autoacidification and temperature downshift in skim milk resulted in the activation of unexpected peripheral pathways upstream of the supposed metabolic bottlenecks (Raynaud, et al. 2005). As far as we know the use of 'omics' for deciphering bacterial metabolism during fermentation has not yet been reported for products other than fermented milk. Indeed, it is still a challenge to study the functions of starters in complex and contaminated food matrices such as meat, sauerkraut or pickles. These matrices cannot be easily sterilized without denaturing their properties. The only articles referring to 'omics' in food are based on liquid fermented products (beer, wine, milk) simply because it is possible to get a filtered or heat sterilized model. Solid food models are still waiting for other reliable methods (gamma sterilization, use of antibiotics).

4 Conclusion: The Dream of Future Applications for 'Omics' in Food

Several studies performed on food models other than those cited above, may provide new insights that should be reasonably accessible in the near future and should be considered as we go forward studying the field of fermented food products. These insights can be classified in two categories: first, ideas or concepts directly derived from basic research that should be applicable to more applied fields or to complex systems, and second, some experimental results obtained with other food organisms or other environments, that should also be exemplified in fermented food matrices.

In the first category the literature offers interesting results with the work of Mandenius (2004), König, et al. (2006) and Izawa, et al. (2006). These authors propose tools to analyze biological data obtained from 'omics' studies, for the modeling of complex biological systems. Although yet obscure as directly applicable tools for food fermentation, these might be considered as the premise for future methods that may help to understand and control complex fermentation ecosystems.

The second category is illustrated by experiences performed on fermented beverages. Wine, Sausage or cheese making encompass the succession of complex microbiological and biochemical phenomena whose complete performance is required to ensure the final quality of the product in terms of safety and organoleptic properties. Global analyzing methods, as available through 'omics,' should represent choice tools that offer insight into the right technological performance throughout the industrial process. Such an approach has been recently reported in the field of fermentation (Hansen, et al. 2006). The authors report the use of proteomics to follow industrial grain fermentation by *Sacharomyces cerevisiae*. They could show increasing amounts of proteins involved in protection against stress and nitrogen limitation all along the fermentation process. The use of 'omics' was also reported as a tool to follow the dynamics of the process of fermentation during the various steps of beer fermentation (Kobi, et al. 2004), and a tool to understand the adaptation of *S. cerevisiae* to wine fermentation (Zuzuarregui, et al. 2006). Alternative strategies have also been developed to detect genes that are specifically expressed in some conditions. The in vivo expression technology (IVET) has been successfully used with a strain of *Lactobacillus reuteri*, a lactic acid bacterium isolated from the digestive tract of a rat. In this study, the genes that were specifically induced in the mouse gastrointestinal tract were identified (Walter, et al. 2003). Later, the same approach was used to identify genes specifically induced in *Lb. reuteri* during sourdough fermentation (Dal Bello, et al. 2005). This method is applicable to many complex fermented systems. Indeed, 15 *in carnis* induced genes of *Lb. sakei* have been detected by IVET, showing that during Sausage fermentation, this species specifically induces genes related to stress response and to ammonia acquisition (Hüfner, et al. 2007). However, although very informative to detect

genes whose expression is specifically induced in such complex food processes, the method has a limitation, simply because the genes that are also expressed in laboratory conditions, and may be important for fermentation process, are ignored during the construction of the IVET library. It may, however, be an interesting tool to use as a complement to other global approaches.

Another field that is still difficult to assess, but that should largely benefit in the future from the consequences of the 'omics,' is a better characterization of the bacterial ecosystems that are composing fermented foods. It has long been known that a succession of various bacterial species often occurs during fermentation processes. For instance in fermented Sausages, it has been shown that the species, and even the strains, are not present at the same level throughout the process (Cocolin, et al. 2001; Ammor, et al. 2005). Several accurate methods exist that allow an efficient evaluation and monitoring of the bacterial species present in a complex fermented food. In particular, methods that do not require selection and plating of bacteria, but instead use molecular approaches and avoid a culture step, have been developed (Giraffa and Neviani 2001). They allow estimation of which bacterial population is present in a complex environment. Recently, Martin, et al. (2006) could specifically detect and quantify the *Lb. sakei* population in meat and fermented Sausages by quantitative real-time PCR. However, until now, those detection methods could not differentiate strains of the same species. One can expect that, in the near future, genome data obtained from different strains belonging to a same species will allow scientists to discriminate strains and, thus, allow monitoring of individual strains. One can imagine that such an accuracy could be useful to monitor and track bacteria as well as processes. For instance, this could be used to assess the properties or traits of various bacterial strains, to characterize various fermentation processes or steps, for the early detection of possible unwanted by-products during fermentation, or for the quality control of the final product. Such accurate methods could also be developed for the traceability of processed food products and the Protected Designation of Origin (PDO) traditional fermented foods.

References

- Altermann, E., Russel, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., Klaenhammer, T.R., 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proc. Natl. Acad. Sci. USA* 102, 3906–3912.
- Ammor, S., Dufour, E., Zagorec, M., Chaillou, S., Chevallier, I., 2005. Characterization and selection of *Lactobacillus sakei* strains isolated from traditional dry sausage for their potential use as starter cultures. *Food Microbiol.* 22, 529–538.
- Åvall-Jääskeläinen, S., Palva, A., 2005. *Lactobacillus* surface layers and their applications. *FEMS Microbiol. Rev.* 29, 511–529.
- Bae, J.-W., Rhee, S.-K., Park, J.R., Chung, W.-H., Nam, Y.-D., Lee, I., Kim, H., Park, Y.-H., 2005. Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. *Appl. Environ. Microbiol.* 71, 8825–8835.
- Boekhorst, J., Siezen, R.J., Zwahlen, M.-C., Vilanova, D., Pridmore, R.D., Mercenier, A., Kleerebezem, M., de Vos, W.M., Brüssow, H., Desiere, F., 2004. The complete genomes of

- Lactobacillus plantarum* and *Lactobacillus johnsonii* reveal extensive differences in chromosome organization and gene content. *Mirobiology* 150, 3601–3611.
- Boekhorst, J., Helmer, Q., Kleerebezem, M., Siezen, R.J., 2006. Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiology* 152, 273–280.
- Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarne, K., Weissenbach, J., Ehrlich, S.D., Sorokin, A., 2001. The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *Lactis* IL1403. *Genome Res.* 11, 731–753.
- Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S.D., Kulakauskas, S., Lapidus, A., Goltsman, E., Mazur, M., Pusch, G.D., Fonstein, M., Overbeek, R., Kyprides, N., Purnelle, B., Prozzi, D., Ngui, K., Masuy, D., Hancy, F., Burteau, S., Boutry, M., Delcour, J., Goffeau, A., Hols, P., 2004. Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nat. Biotechnol.* 22, 1554–1558.
- Chaillou, S., Champomier-Vergès, M., Cornet, M., Crutz Le Coq, A.-M., Dudez, A.-M., Martin, V., Beaufils, S., Darbon-Rongère, E., Bossy, R., Loux, V., Zagorec, M., 2005. Complete genome sequence of the meat-borne lactic acid bacterium *Lactobacillus sakei* 23K. *Nat. Biotechnol.* 23, 1527–1533.
- Claesson, M.J., Li, Y., Leahy, S., Canchaya, C., van Pijkeren, J.P., Cerdeño-Tárraga, A.M., Parkhill, J., Flynn, S., O'Sullivan, G.C., Collins, J.K., Higgins, D., Shanahan, F., Fitzgerald, G.F., van Sinderen, D., O'Toole, P.W., 2006. Multireplicon genome architecture of *Lactobacillus salivarius*. *Proc. Natl. Acad. Sci. USA* 103, 6718–6723.
- Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages. *Appl. Environ. Microbiol.* 67, 5113–5121.
- Coenye, T., Gevers, D., Van de Peer, Y., Vandamme, P., Swings, J., 2005. Towards a prokaryotic genomic taxonomy. *FEMS Microbiol. Rev.* 29, 147–167.
- Dal Bello, F., Walter, J., Roos, S., Jonsson, H., Hertel, C., 2005. Inducible gene expression in *Lactobacillus reuteri* LTH5531 during type II sourdough fermentation. *Appl. Environ. Microbiol.* 71, 5873–5878.
- Derzelle, S., Bolotin, A., Mistou, M.-Y., Rul, F., 2005. Proteome analysis of *Streptococcus thermophilus* grown in milk reveals pyruvate formate-lyase as the major upregulated protein. *Appl. Environ. Microbiol.* 71, 8597–8605.
- Doolittle, W.F., Papke, R.T., 2006. Genomics and the bacterial species problem. *Genome Biol.* 7, 116.
- Fleischman, R.D., Adams, M.D., White, O., Clayton, R.A., Kirkness, E.F., Kerlavage, A.R., Bult, C.J., Tomb, J.F., Dougherty, B.A., Merrick, J.M., McKenney, K., Sutton, G., FitzHugh W., Fields, C., Gocayne, J.D., Scott, J., Shirley, R., Liu, L.-I., Glodek, A., Kelley, J.M., Weidman, J.F., Phillips, C.A., Spriggs, T., Hedblom, E., Corron, M.D., Utterback, T.R., Hanna, M.C., Nguyen, D.T., Saudek, D.M., Brandon, R.C., Fine, L.D., Fritchman, J.L., Fuhrmann, J.L., Geoghagen, N.S.M., Gnehm, C.L., McDonald, L.A., Small, K.W., Fraser, C.M., Smith, H.O., Venter, C.J., 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496–512.
- Gagnaire, V., Piot, M., Camier, B., Vissers, J.P.C., Jan, G., Léonil, S., 2004. Survey of bacterial proteins released in cheese: a proteomic approach. *Int. J. Food Microbiol.* 94, 185–201.
- Giraffa, G., Neviani, E., 2001. DNA-based, culture-independent strategies for evaluating microbial communities in food-associated ecosystems. *Int. J. Food Microbiol.* 67, 19–34.
- Gitton, V., Meyrand, M., Wang, J., Caron, C., Trubuil, A., Mistou, M.-Y., 2005. Proteomic signature of *Lactococcus lactis* NCDO763 cultivated in milk. *Appl. Environ. Microbiol.* 71, 7152–7163.
- van de Guchte, M., Penaud, S., Grimaldi, C., Barbe, V., Bryson, K., Nicolas, P., Robert, C., Oztas, S., Mangenot, S., Couloux, A., Loux, V., Dervyn, R., Bossy, R., Bolotin, A., Batto, J.-M., Walunas, T., Gibrat, J.-F., Bessières, P., Weissenbach, J., Ehrlich, S. D., Maguin, E., 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proc. Natl. Acad. Sci. USA* 103, 9274–9279.

- Hammes, W.P., Hertel, C., 2004. The genera *Lactobacillus* and *Carnobacterium*. In: S.F.M. Dworkin, E. Rosenberg, K.H. Schleiffer and E. Sackebrandt (Eds.), *The Prokaryotes: an evolving electronic resource for the microbiological community*, release 3.15. Springer, New York.
- Hansen, R., Pearson, S.Y., Brosnan, J.M., Meaden, P.G., Jamieson, D.J., 2006. Proteomic analysis of a distilling strain of *Saccharomyces cerevisiae* during industrial grain fermentation. *Appl. Microbiol. Biotechnol.* 72, 116–125.
- Hols, P., Hancy, F., Fontaine, L., Grossiord, B., Prozzi, D., Leblond-Bourget, N., Decaris, B., Bolotin, A., Delorme, C., Ehrlich, S.D., Guédon, E., Monnet, V., Renault, P., Kleerebezem, M., 2006. New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiol. Rev.* 29, 435–463.
- Hollywood, K., Brison, D.R., Goodacre, R., 2006. Metabolomics: current technologies and future trends. *Proteomics* 6, 4716–4723.
- Hüfner, E., Markieton, T., Chaillou, S., Crutz-Le Coq A.-M., Zagorec, M., Hertel, C. 2007. Identification of *Lactobacillus sakei* genes induced during meat fermentation and their role in survival and growth. *Appl. Environ. Microbiol.* 73, 2522–2531.
- Izawa, N., Kishimoto, M., Konishi, M., Omasa, T., Shioya, S., Ohtake, H., 2006. Recognition of culture state using two-dimensional gel electrophoresis with an artificial neural network. *Proteomics* 6, 3730–3738.
- Klaenhammer, T.R., Barrangou, R., Buck, B.L., Azcarate-Peril, M.A., Altermann, E., 2005. Genomic features of lactic acid bacteria effecting bioprocessing and health. *FEMS Microbiol. Rev.* 29, 393–409.
- Kleerebezem, M., Boekhorst, J., van Kranenburg, R., Molenaar, D., Kuipers, O.P., Leer, R., Tarchini, R., Peters, S.A., Sandbrink, H.M., Flers, M.W.E.J., Stiekema, W., Klein Lankhorst, R.M., Bron, P.A., Hoffer, S.M., Nierop Groot, M.N., Kerkhoven R., de Vries, M., Ursing, B., de Vos, W.M., Siezen, R.J., 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proc. Natl. Acad. Sci. USA* 100, 1990–1995.
- Kobi, D., Zugmeyer, S., Potier, S., Jaquet-Gutfreund, L., 2004. Two-dimensional protein map of an „ale“-brewing yeast strain: proteome dynamics during fermentation. *FEMS Yeast Res.* 5, 213–230.
- Kok, J., Buist, G., Zomer, A.L., van Hijum, S.A.F.T., Kuipers, O.P., 2005. Comparative and functional genomics of lactococci. *FEMS Microbiol. Rev.* 29, 411–433.
- König, R., Schramm, G., Oswald, M., Seitz, H., Sager, S., Zapatka, M., Reinelt, G., Eils, R., 2006. Discovering functional gene expression patterns in the metabolic network of *Escherichia coli* with wavelets transforms. *BMC Bioinformatics* 7, 119.
- Lan, R., Reeves, P.R., 2000. Intraspecies variation in bacterial genomes: the need for a species genome concept. *Trends Microbiol.* 8, 396–401.
- Larsen, N., Boye, M., Siegumfeldt, H., Jakobsen, M., 2006. Differential expression of proteins and genes in the lag phase of *Lactococcus lactis* subsp. *Lactis* grown in synthetic medium and reconstituted skim milk. *Appl. Environ. Microbiol.* 72, 1173–1179.
- Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.-H., Diaz-Muñiz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O’Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kosyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. *Proc. Natl. Acad. Sci. USA* 103, 15611–15616.
- Mandenius, C. F., 2004. Recent developments in the monitoring, modeling and control of biological production systems. *Bioprocess Biosyst. Eng.* 26, 347–351.
- Martin, B., Jofré, A., Garriga, M. Pla, M., Aymerich, T. 2006. Rapid quantitative detection of *Lactobacillus sakei* in meat and fermented sausages by real-time PCR. *Appl. Environ. Microbiol.* 72, 6040–6048.

- Molenaar, D., Bringel, F., Schuren, F.H., de Vos, W.M., Siezen, R.J., Kleerebezem, M., 2005. Exploring *Lactobacillus plantarum* genome diversity by using microarrays. *J. Bacteriol.* 187, 6119–6127.
- Park, S. J., Lee, S. Y., Cho, J., Kim, T. Y., Lee, J. W., Park, J. H., Han, M. J., 2005. Global physiological understanding and metabolic engineering of microorganisms based on omics studies. *Appl. Microbiol. Biotechnol.* 68, 567–579.
- Pessione, E., Mazzoli, R., Giuffrida, M. G., Lamberti, C., Garcia-Moruno, E., Barello, C., Conti, A., Giunta, C., 2005. A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics* 5, 687–698.
- Pool, W.A., Neves, A.R., Kok, J., Santos, H., Kuipers, O.P., 2006. Natural sweetening of food products by engineering *Lactococcus lactis* for glucose production. *Metab. Eng.* 8, 456–464.
- Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, A.-C., Zwahlen, M.-C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Kleanhammer, T.R., Arigoni, F., Schell, M.A., 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. *Proc. Natl. Acad. Sci. USA* 101, 2512–2517.
- Raynaud, S., Perrin, R., Coccagn-Bousquet, M., Loubière, P., 2005. Metabolic and transcriptomic adaptation of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis in response to autoacidification and temperature downshift in skim milk. *Appl. Environ. Microbiol.* 71, 8016–8023.
- Schachtsiek, M., Hammes, W.P., Hertel, C., 2004. Characterization of *Lactobacillus coryniformis* DSM 20001^T surface protein Cpf mediating co-aggregation with an aggregation among pathogens. *Appl. Environ. Microbiol.* 70, 7078–7085.
- Schell, M.A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M.-C., Desiere, F., Bork, P., Delley, M., Pridmore, R.D., Arigoni, F., 2002. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal. *Proc. Natl. Acad. Sci. USA* 99, 14422–14427.
- Siezen, R.J., Boekhorst, J., Muscariello, L., Molenaar, D., Renckens, B., Kleerebezem, M. 2006. *Lactobacillus plantarum* gene clusters encoding putative cell surface protein complexes for carbohydrate utilization are conserved in specific gram positive bacteria. *BMC Genomics* 7, 126.
- Tannock, G.W., 2004. A special fondness for lactobacilli. *Appl. Environ. Microbiol.* 70, 3189–3194.
- Teusink, B., van Enckevort, F.H.J., Francke, C., Wiersma, A., Wegkamp, A., Smid, E.J., Siezen, R.J., 2005. *In silico* reconstruction of the metabolic pathways of *Lactobacillus plantarum*: comparing predictions of nutrient requirements with those from growth experiments. *Appl. Environ. Microbiol.* 71, 7253–7262.
- Teusink, B., Smid, E.J., 2006. Modelling strategies for the industrial exploitation of lactic acid bacteria. *Nat. Rev. Microbiol.* 4, 46–56.
- Walter, J., Heng, N.C.K., Hammes, W.P., Loach, D.M., Tannock, G.W., Hertel, C., 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. *Appl. Environ. Microbiol.* 69, 2044–2051.
- Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, C., Canchaya, C., Ventura, M., Goesmann, A., Gasson, M.J., Kuipers, O.P., van Sinderen, D., Kok, J., 2007. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. *J. Bacteriol.* 189, 3256–3270.
- Zuzuarregui, A., Monteoliva, L., Gil, C., de Olmo, M., 2006. Transcriptomic and proteomic approach for understanding the molecular basis of adaptation of *Saccharomyces cerevisiae* to wine fermentation. *Appl. Environ. Microbiol.* 72, 836–847.

Index

A

- Acetic acid bacteria (AAB), 169, 171, 176, 178, 181
- Amplified fragment length polymorphism (AFLP), 20, 73
 - in sourdough LAB, 130
- Adjuncts in dairy products, 41–42
- Aggregation promoting factors (APF), 263–264
- Ale-fermenting yeast, 202
- Almagro eggplants fermentation, 150–153

B

- Bacteria, 6
 - adaptation, 3–4
 - flora, 2
 - genome sequence, 255–257
 - microbial communication, 2–3
 - phyla, 225
- Bacterial DGGE
 - gels analyzing fermented Sausages, 98
 - profiles of fermented Sausages, 97
- Bayesian statistics, use of, 248
- Beer
 - making process, 193
 - maturation, 196
- Bioinformatics, 22, 76
- Biomolecular methods, 196–197
- Biotyping methods, 59, 61
- Brewer's yeast, 201–202
- Brewing process, 194–195

C

- Caper berries fermentation, 146–150
- Cassava-based fermented foods, 215–216
- Cell
 - density, 3

- sorting, 12
- surface complexes, 263

Cheeses

- classification, 32–33
- coagulation time, 35–36
- and FISH, 56–58
- making process, 32–33
- microbial ecology, 37–38
- microbial populations, 51–55
- molding, 36
- protected denomination of origin (PDO), 33–34
- raw milk, 34
- rennet, 35–36
- ripening, 37
- salt addition, 36–37
- starter addition, 34–35
- CLUSTAL approach, 247
- Cluster analysis of bacterial and yeast profiles
 - of fermented Sausages, 100
- Coagulase-negative cocci (CNC)
 - and culture-dependent methods, 101–103
 - in Sausage fermentation, 91–93, 109–111
 - from Tucuman Sausages, 100
- Cocoa fermentation, 219–220
- Coffee fermentation, 220–221
- Colony hybridization, 13–15
- Community DNA/RNA isolation approach, 4
- Culture-dependent methods, 212
 - in caper berries fermentation, 149–150
 - LAB and CNC isolated in Sausage fermentation, 104–106
 - in Sausage fermentation, 101–103
 - in wine fermentation, 176–177
- Culture-dependent techniques, 13
- Culture-independent techniques
 - in caper berries fermentation, 147–148
 - in kimchi, 217
 - for microbial analyses, 33

Culture-independent techniques (*cont.*)
 microbial ecology application, 5–6
 in Pozol, 210, 212–214
 in Sausage fermentation, 93–101
 sourdough LAB, 125–127
 sourdough yeasts, 127–128
 species/genus identification, 47–48
 weaknesses, 23
 in wine fermentation, 169–171
 Culture media of probiotic studies, 241
 Curd cooking and cutting, 36

D

Defined strain starters (DSS), 34–35
 DGGE methodology in probiotic studies, 230
 Diagnostic microbiology, 2
 Dairy bacteria, typing of, 60
 Dairy products
 diversity of, 31–34, 37–38, 47
 microbial profiles, 55–56
 microbiological aspects of, 31–34
 molecular methods, 47
 molecular tools, 78–79
 production phases, 34–37
 Direct epifluorescence technique (DEFT), 171
 Deoxyribonucleic acid (DNA)
 based identification techniques, 58–59, 61
 based typing, 18, 128–129
 chip microarray technology, 21
 digests, 238
 DNA Hybridization Methods, 13–15
 extraction, 47–48
 fingerprinting, brewing yeasts, 201
 fragments, 6–8
 hybridization, 262–263
 PFGE of, 238–240
 probes, 15, 228–229, 232
 sequencing, 16–17, 22, 249–252
 Dot-blot hybridization, 12, 15

E

Ecosystems, 3
 Element sequence-based PCR. *See* Rep-PCR
 Enterococci
 in fermented Sausages, 102
 molecular approaches, 67
Enterococcus faecalis, 3
 Ethanol production, 168
 Evolutionary relatedness, 246–249

F

Fermentation
 alcoholic, 145–146
 Almagro eggplants, 150–153
 caper berries, 146–150
 of dairy products, 31–34
 milk, 145–146, 267–268
 processes, 269–270
 reactions, 245–246
 sauerkraut, 153–154
 table olives, 154–156
 vegetable, 145–146, 156–157
 Fermented foods, 208–210
 cassava-based, 215–216
 consumption in various countries, 211–212
 maize-based, 210, 212–214
 milks, 32
 perspectives, 221–222
 sorghum-based, 214–215
 Fluorescent AFLP (FAFLP), 73
 5'Fluorogenic exonuclease (TaqMan) assay.
 See TaqMan
 Flow cytometry (FCM), 11–12, 33, 171
 Fluorescence in situ hybridization (FISH)
 in mesophilic starter culture, 33
 microbial colonies in cheese, 56–58
 probiotic studies, 232–235
 of Stilton cheese sections, 57
 uses of, 10–11
 in wine fermentation, 171–172
 Fluorescence-labeled primer technology, 9
 Food
 ecology, 3
 ecosystems, 4
 products, genomic diversity, 262

G

2-D Gel electrophoresis, 202
 Gene
 characterization, 134–135
 cloning, 134–135
 probes, 128
 regulation, 135
 diversity in Almagro eggplant fermentation, 152–153
 Genome probing microarray (GPM), 21, 218
 Genome-scale modeling methods of LAB, 263–264, 266
 Genotypic methods, 17, 59
 Genus- and species-specific PCR assays, 77–78

Global analyzing methods, 269–270
GRAM negative and GRAM positive
microbial species, 3

H

Human Genome Project, 22
Human large bowel, 225–226
Hybridization
 of bulk DNA, 21
 DNA-DNA methods, 13–15
 dot-blot, 12, 15
 methods, 212–213
 probiotic studies, 233–234
 procedure in cheese, 56–58
 quantitative RNA method, 212–213
 reverse dot-blot, 12
 RNA method, 212–213
 in situ, 10–11

I

In silico biology, 22
In-situ methods, 9–11
In-situ PCR, 11
In-situ reactions, 3–4
In vivo metabolism, 267
Italian dairy tradition, 36

J

Japanese fermentation process, 216–217

K

Korean fermented food (Kimchi), 217–219

L

Lactic acid bacteria (LAB)
 adhesion properties of, 263–265
 in beer fermentation, 203–206
 biodiversity of, 149
 cabbage, 153
 in caper berries fermentation, 148
 in cheeses, 54
 cultivable microflora, 58
 genome annotation, 265–266
 genome sequences, 258–259
 genomic biodiversity, 262–263
 genomic diversity, 260–263
 genomics, 256–259
 green olives, 155
 introduction of, 2–3

 isolated from sourdoughs, 121–122
 metabolic engineering, 267
 metabolic pathways, 265–266
 molecular approaches, 62–63
 new species of, 59
 in Sausage fermentation, 91–93, 100,
 107–109
 in silico analysis, 265–266
 sorghum isolates, 214
 in sourdough, 131–132
 spoilage in beer, 203–206
 in table olives fermentation, 154–156
 taxonomy in sourdough, 120, 122–123
 use of DNA probes, 15
 in wine fermentation, 168–169

Lactobacilli

 alignment based phylogeny, 248, 250
 in Almagro eggplant fermentation, 151
 molecular approaches, 64
 in sourdough LAB, 134–135

Lactococci molecular approaches, 66

Length heterogeneity-PCR (LH-PCR), 8, 127

Leuconostocs, molecular approaches, 68

M

Maize-based fermented foods, 210, 212–214

Malting process, 194–195

Meat fermentation, 91–93

Metabolic modeling, 265–267

Metabolites, 2, 167

Metabolomics, 256, 264–265

Microbial cultures, 35

 analyses, 2

 communications, 3–4

 community comparison tools, 252

 diversity, 6

Microbial ecology, 226

 of cheese, 37–38

 culture-independent techniques

 applications, 5–6

 in dairy fermentation, 33

 of food products, 262–263

 in Sausage fermentation, 92–94

 of winemaking process, 163–167

Microbial populations

 of caper fermentation, 147, 149–150

 in cheeses, 33, 36, 51–55

 data, quantitative, 3

 in fermented foods, 2–4

 identification, 13

 profiles, 125–127

 in Salers cheese, 54

 types, 17, 19

- in wine fermentation, 169–171
 - Microbiota analysis, 245–246
 - cassava, 215–216
 - challenge, 252–253
 - diversity, 246–250
 - Microflora, 2
 - in cheeses, 32–33
 - cultivable, 59, 61
 - femented Sausage, 101–103
 - in fermented Sausages, 107, 110–111
 - of grapes, 167
 - in Sausage fermentation, 92, 94, 97
 - in sourdough LAB, 130
 - Microorganisms, 36
 - adjuncts, 41–42
 - in beer fermentation, 196–197
 - biological activity of, 145–146
 - and evolutionary relatedness, 246–249
 - in food fermentation, 2–4
 - molecular differentiation of, 22–23
 - in natural fermentations, 210
 - non-starter, 42–46
 - in PCR-based methods, 8–9
 - and salting, 37
 - starters, 39–41
 - study approaches, 47
 - in wine fermentation, 165–167
 - Milk
 - clotting, 35–36
 - fermentation, 268
 - pre-treatment, 34
 - standardization, 34
 - Mito-RFLP method, 179
 - Mixed strain starters (MSS), 34–35
 - Molecular identification methods, 13
 - Molecular methods
 - advantages and disadvantages of, 14
 - analysis techniques, 132–133
 - application of, 156–157
 - brewing yeasts, 197–198
 - chronometers, 246–247
 - cultivable microflora of dairy products, 61
 - data analysis, 22
 - in dairy microbiology, 78–79
 - and fermented foods, 210, 212–216
 - limitations of, 22–23
 - overview of, 2–3
 - within polyphasic approaches, 33–34
 - scientific literature, 107
 - sourdough ecology, 132
 - sourdough LAB, 128–130, 135–136
 - sourdough yeasts, 130–131
 - soybean and rice, 216–217
 - Molecular typing methods, 129
 - Mucin binding proteins (MUB), 263
 - Multicolor FISH, 11
 - Multi-locus hybridization typing (MLHT), 74
 - Multi-locus restriction typing (MLRT), 74
 - Multi-locus sequence analysis (MLSA), 125
 - Multi-locus sequence typing (MLST), 74
 - Multimer transformation, principle of, 250
 - Multiple probe concept, 15
 - Multiple sequence alignment, 247–249
 - Multiplex FISH, 11
 - Multiplex PCR, 16, 77, 126, 174
- N**
- Natural whey cultures (NWC), 35, 48–51
 - Non-starter microorganisms, 42–46
 - Nucleic acid-based analytical methods, 227–229
 - flow chart, 229
 - Nucleic acid-based methods, 240–242
 - in human large bowel, 225–226, 228–229
 - in wine fermentation, 171–172
 - Nucleotide probes, 178
- O**
- Oligonucleotide probes, 10, 12–13, 232
 - Operational taxonomic units (OTU), 226
- P**
- Pathogens, 3
 - PCR amplification
 - in dairy samples, 53–55
 - of DNA, 9, 23
 - in microbial communities, 7–8
 - protocols, 16
 - species/genus identification, 47–48
 - PCR-based methods
 - in beer fermentation, 197–202
 - DNA fingerprinting, 18–19, 72
 - in microbial ecology, 4, 6–9
 - and molecular techniques, 15–16
 - value in estimating strains, 156–157
 - in wine fermentation, 174–175
 - PCR-denaturing gradient gel electrophoresis (PCR-DGGE)
 - application in Sausage fermentation, 99–101
 - application of, 6–7
 - cassava-based fermented foods, 215–216
 - in cocoa fermentation, 219–220
 - in dairy products, 55–56
 - fingerprinting, 48–51, 156–157, 213–214

- in kimchi, 218–219
- probiotic studies, 229–232
- in Sausage fermentation, 94–97
- sequence polymorphism, 74
- in starter cultures, 127–128
- in stilton cheese, 52
- for strain identification, 109
- in wine fermentation, 172–174
- in wine yeast isolates, 178
- PCR-RFLP, 76
- PCR-temperature gradient gel electrophoresis (PCR-TGGE)
 - application of, 6–7
 - in caper berries fermentation, 147–150
 - in dairy products, 52–53
 - probiotic studies, 229–232
 - in Sausage fermentation, 94
- Phenotypic methods, 13, 59, 61
- Phylogenetic group, 256–257
 - markers, 7
 - in sourdough LAB, 123–125
- Phylogenetic tree construction, 247–248, 252
- Plasmid profiling, 129
- Plate culturing techniques, 3
- Polymerase chain reaction (PCR)
 - assays, 128
 - based fingerprinting methods, 156–157
 - bias, 8
 - screens in wine fermentation, 179
- Polyphasic ecology approach, 9
- Pozol, 210, 212–214
- Principal component analysis (PCA), 249–250
- Probiotic studies, 229–230
- Propionibacteria, molecular approaches, 69
- Protected denomination of origin (PDO)
 - cheeses, 33–34
- Proteomics, 264–265, 267–269
- Pulse-field gel electrophoresis (PFGE), 238–240
 - in sourdough LAB, 129
 - in probiotic studies, 239
- Q**
- Quantitative hybridization approach, 12
- Quantitative PCR (qPCR), 20–22, 33, 175–176
- Quorum Sensing (QS), 3–4
- R**
- Random amplified polymorphic DNA (RAPD)
 - analysis, 108–109
 - in sourdough LAB, 130
- RAPD-PCR
 - fingerprinting in soybean, 216
 - in food ecosystems, 18–20
 - primers, dairy microorganisms, 70–71
 - sauerkraut fermentation, 153–154
 - Sausage fermentation, 110
 - sourdough LAB, 129–130
 - strain typing, 61, 68–69
 - in wine fermentation, 176
- Real time PCR, 20
- REA-PFGE, 18, 72–75
- REA-pulsed field gel electrophoresis, 18
- REA/RFLP, in sourdough LAB, 129
- Relative quantification, 175–176
- Rennet cheeses, 35–36
- Rep-PCR, 19
- Restriction endonuclease analysis (REA), 18
- Restriction fragment length polymorphism (RFLP)-based methods, 18, 23
- Reverse dot-blot hybridization, 12
- Reverse transcriptase-PCR (RT-PCR), 4
- RiboPrinter system, 18
- Ribotyping, 18, 75
- Ripening technique, in Sausage fermentation, 91–93
- RNA hybridization method, 212–213
- RpoB genes, 7
- rRNA genes, 10–11
 - of DNA, 15–16
 - probes, 103
 - RFLP approaches, 177–178
 - sequence analysis, 13, 177
 - yeast identification, 17
- 16S-23S rRNA gene Intergenic Transcribed Spacer/nl(ITS)-PCR (RISA/ITS-PCR), 9, 16
- 16S rRNA gene sequencing
 - advantages of, 247
 - and bacterial identification, 17
 - encoding, 6–8
 - microflora in starter cultures, 51–52
 - resolving power of, 123–124
 - in Sausage fermentation, 96–101, 103
 - in starter cultures, 48–49
 - strains identification, 76–77
- 16S rRNA probes, 56
- rRNA operon genes, 17
- S**
- Sauerkraut fermentation, 153–154
- Sau-PCR, 73
- Sausage fermentation
 - culture-dependent methods, 101–103
 - culture-independent methods, 95–100
 - future developments in, 111–112

introduction, 91–93
 SDS-PAGE, 76
 in Almagro eggplant fermentation, 152
 of WCPs, 73–74
 Sequence-based identification systems, 17
 Signature sequence, 249
 Single-strand conformation polymorphism
 (SSCP)-PCR analysis (SSCP-PCR
 analysis), 7, 53–55
 Sorghum-based fermented foods, 214–215
 Sourdough fermentation, 119–120
 Sourdough LAB
 gene cloning, 134–135
 genetic amenability of, 133–134
 molecular methods, 128–130
 phylogenetic studies, 123–125
 Sourdough yeasts, 125
 Spatial heterogeneity, 2
 Species-specific identification method, 19,
 75–78
 Species-specific PCR assays, 77–78, 94,
 107–108
 Starter addition, 34–35
 Starter cultures
 in Almagro eggplant fermentation,
 152–153
 development of, 59
 diversity and dynamism of, 48–51
 Starter microorganisms in dairy products,
 39–41
 Strain discrimination in wines, 179–181
 Strains in brewing process, 194–195,
 198–201
 Strain typing, 61, 68–69, 72–74
 Streptococci, molecular approaches, 65
 Synerisis, 36

T

Table olives fermentation, 154–156
 TaqMan, 21

Terminal-Restriction Fragment Length
 Polymorphism (T-RFLP)
 description of, 7–9
 probiotic studies, 236–237
 Traditional fermented foods, 211–212
 Transcriptomic analyses, 256–257, 267–268
 Type-ability, 59
 Typing systems
 dairy bacteria, 60
 sourdough, 131

V

Viable but non-cultivable (VBNC) state, 3,
 169–171

W

Whole genome PCR sampling techniques,
 179, 181–182
 Wine bacteria, 181–182
 Wine fermentation future, 182–183
 Winemaking
 process, 162–165
 general schematic of, 164
 Wine yeast, 179–181
 Wort production, 195–196

Y

Yeasts
 brewing, 201–202
 choice in beer production, 194–195
 in cocoa fermentation, 219–220
 in coffee fermentation, 220–221
 DGGE profiles of fermented Sausages, 99
 dynamics, in fermented Sausages, 102
 ecology in Sausage fermentation, 98–99
 used in sourdough, 125, 130–131
 in wine fermentation, 166–168, 172–174